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Savarino(10) **Patent No.:** **US 9,079,945 B2**
(45) **Date of Patent:** **Jul. 14, 2015**(54) **ADHESIN AS IMMUNOGEN AGAINST
ENTEROTOXIGENIC *ESCHERICHIA COLI***2005/0241024 A1 10/2005 Langridge et al.
2006/0269560 A1 11/2006 Savarino
2007/0237791 A1 10/2007 Ranallo et al.(75) Inventor: **Stephen J. Savarino**, Kensington, MD
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Spring, MD (US)WO WO 01/04148 1/2001
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WO WO 2005/113827 12/2005(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
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11, 2005.(51) **Int. Cl.***A61K 39/00* (2006.01)
A61K 39/385 (2006.01)
A61K 39/108 (2006.01)
A61K 39/02 (2006.01)
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C12P 21/04 (2006.01)
C07K 16/12 (2006.01)
C07K 14/245 (2006.01)(52) **U.S. Cl.**CPC *C07K 16/1232* (2013.01); *A61K 39/0258*
(2013.01); *C07K 14/245* (2013.01); *A61K 38/00*
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2039/545 (2013.01); *C07K 2317/55* (2013.01);
C07K 2319/00 (2013.01); *C07K 2319/35*
(2013.01)(58) **Field of Classification Search**CPC .. *C07K 2319/00*; *C07K 14/245*; *A61K 38/00*;
A61K 39/0258

See application file for complete search history.

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Primary Examiner — Gary Nickol*Assistant Examiner* — Lakia Tongue(74) *Attorney, Agent, or Firm* — McDonnell Boehnen Hulbert
& Berghoff LLP(57) **ABSTRACT**The inventive subject matter relates to the methods for the
induction of immunity and prevention of diarrhea resulting
from *Escherichia coli*. The inventive subject matter also
relates to the use *Escherichia coli* adhesins as immunogens
and to the construction of conformationally stability and pro-
tease resistant *Escherichia coli* adhesin constructs useful for
inducing immunity to *Escherichia coli* pathogenic bacteria.
The methods provide for the induction of B-cell mediated
immunity and for the induction of antibody capable of inhib-
iting the adherence and colonization of *Escherichia coli*
including enterotoxigenic *Escherichia coli*, to human cells.**7 Claims, 12 Drawing Sheets**

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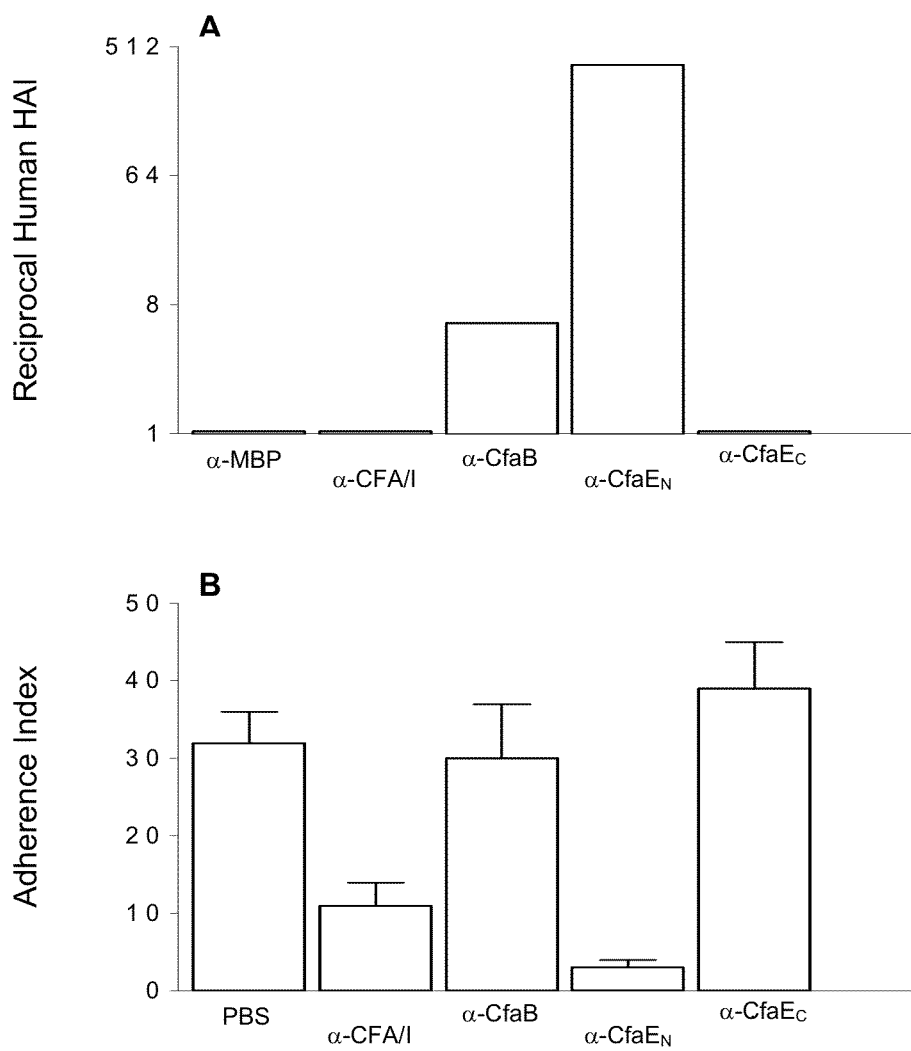


FIG 1

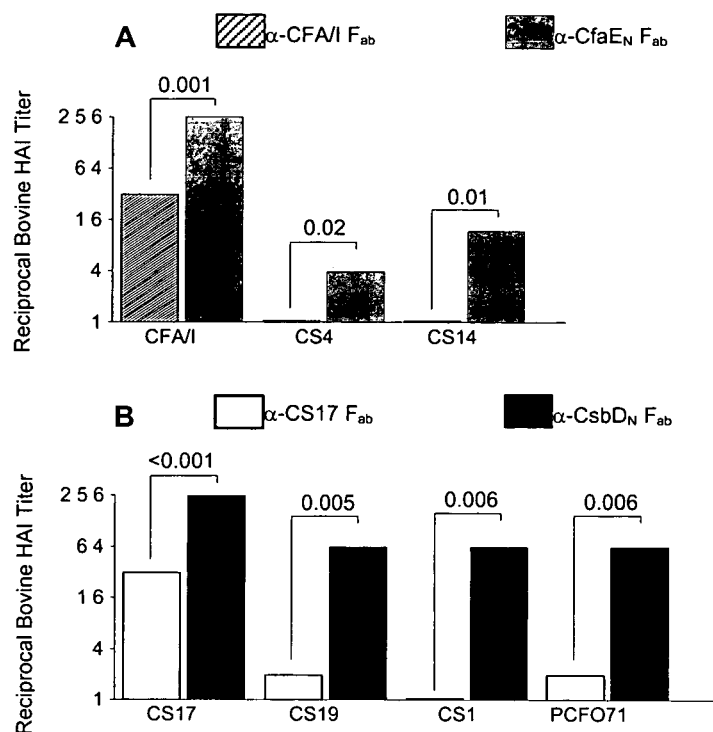


FIG 2

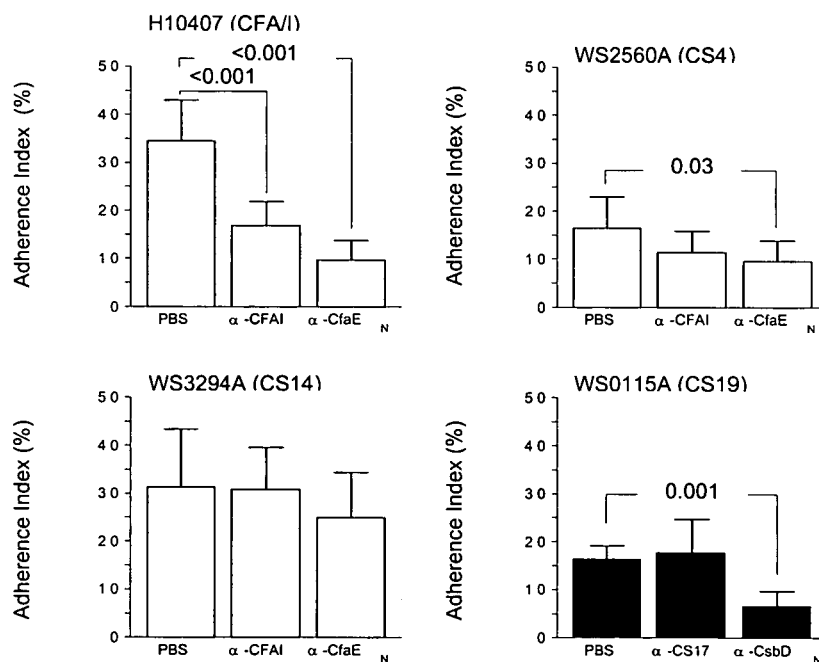


FIG 3

	Major subunit	Fimbriae
VEKNITVTASVDEVIDLIQA	CfaB	CFA/I
VEKNITVTASVDETIDLIQA	CsfA	CS4
VEKNITVTASVDETIDLIQA	CsuA1	CS14
VEKNITVTASVDETIDLIQA	CsuA2	CS14
VEKTLISVTASVDETVDLIQS	CocA	CS1
VEKTLISVTASVDETVDLIQS	CosA	PCFO71
VEKNITVRASVDEKIDLIQA	CsbA	CS17
VEKNITVRASVDEKIDLIQA	CsdA	CS19
AEKNITVTASVDETIDLIQS	CotA	CS2
VOEDITVTANVDTTLEMLSA	CblA	Bcep
VOEDITVTANVDSTLELLQA	TsaB	Styp

UZKxUTUxAxUDxxUDUUxx

FIG 4

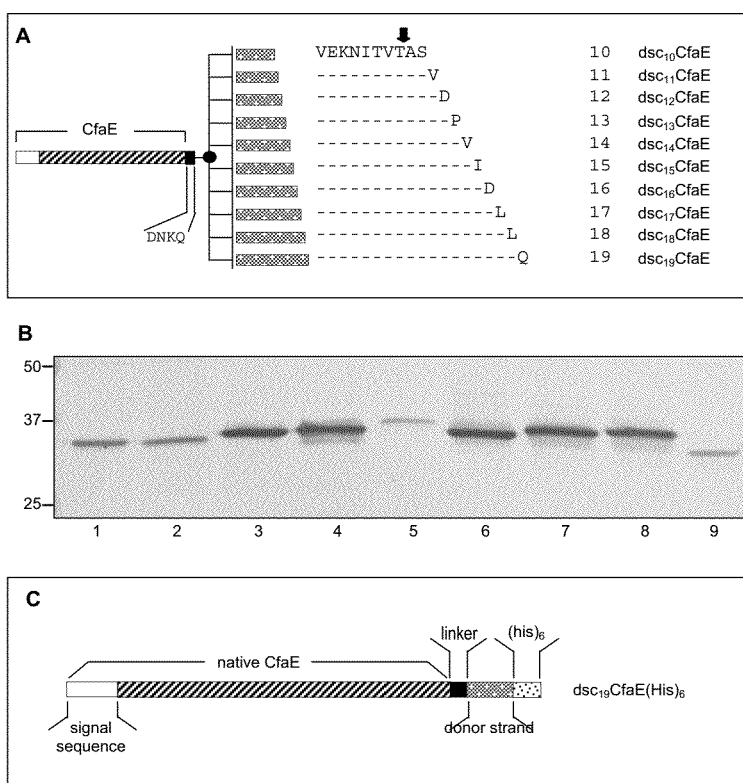


FIG 5

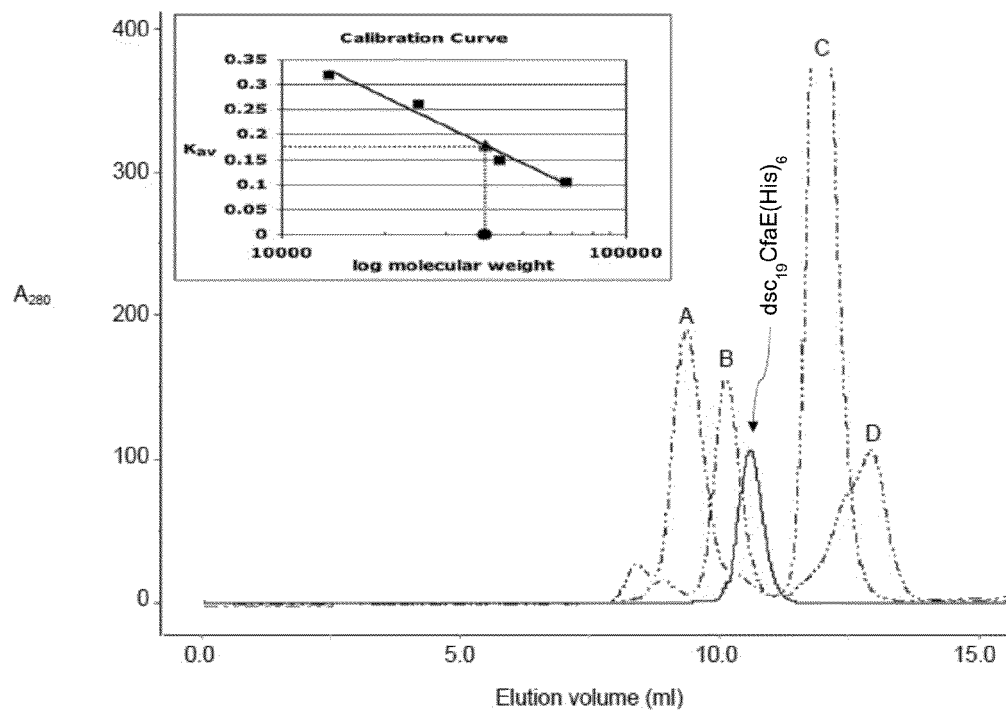


FIG 6

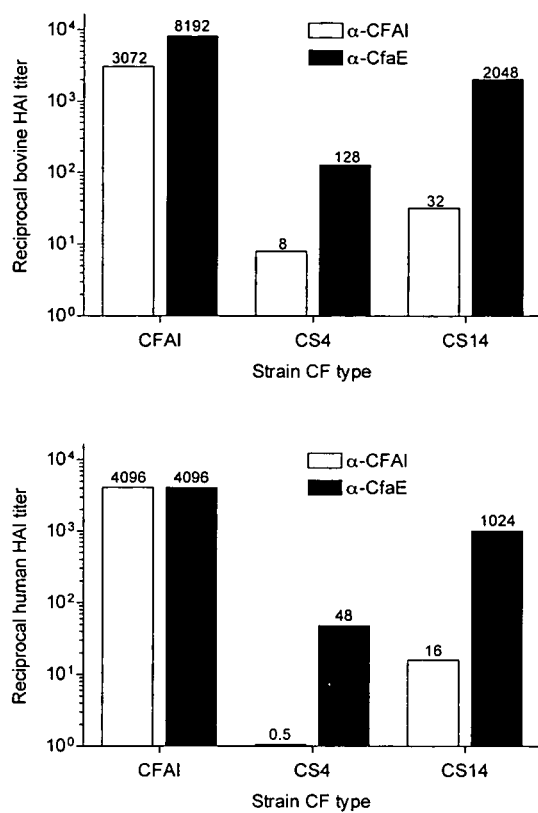


FIG 7

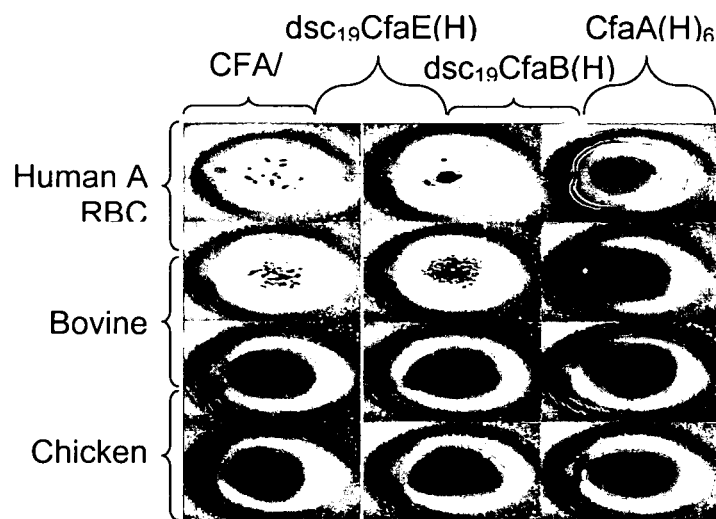


FIG 8

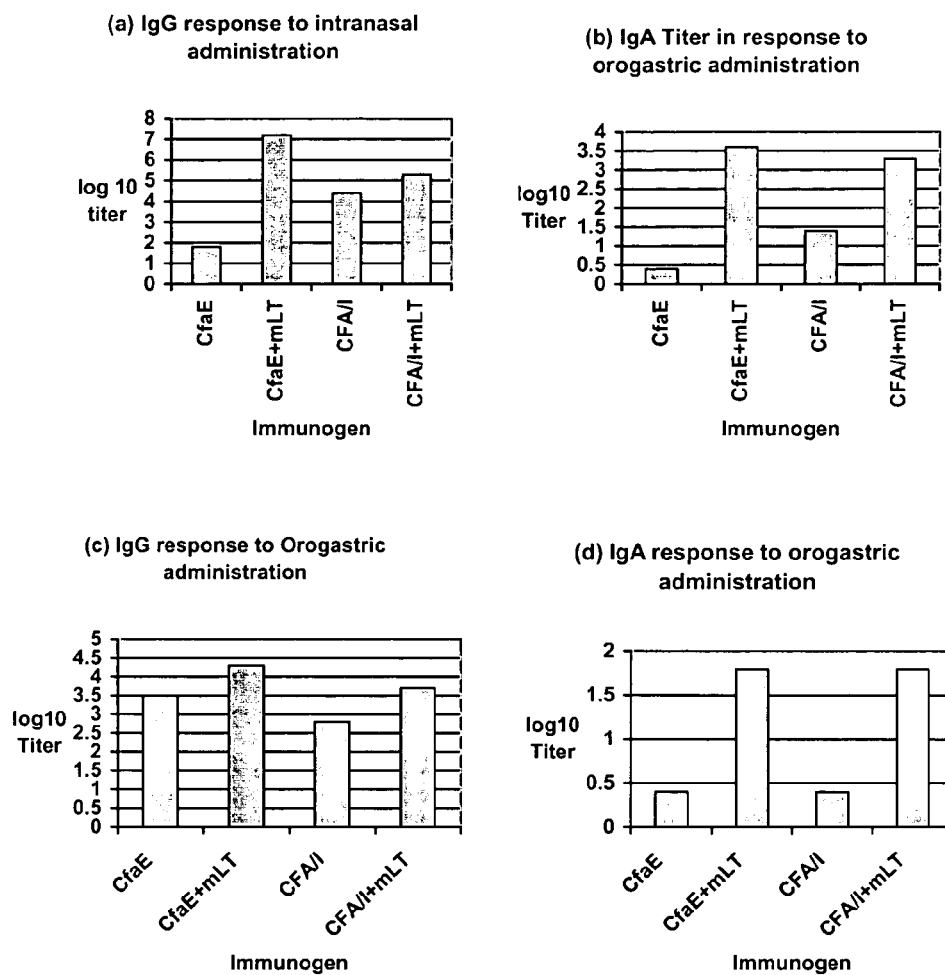


FIG 9

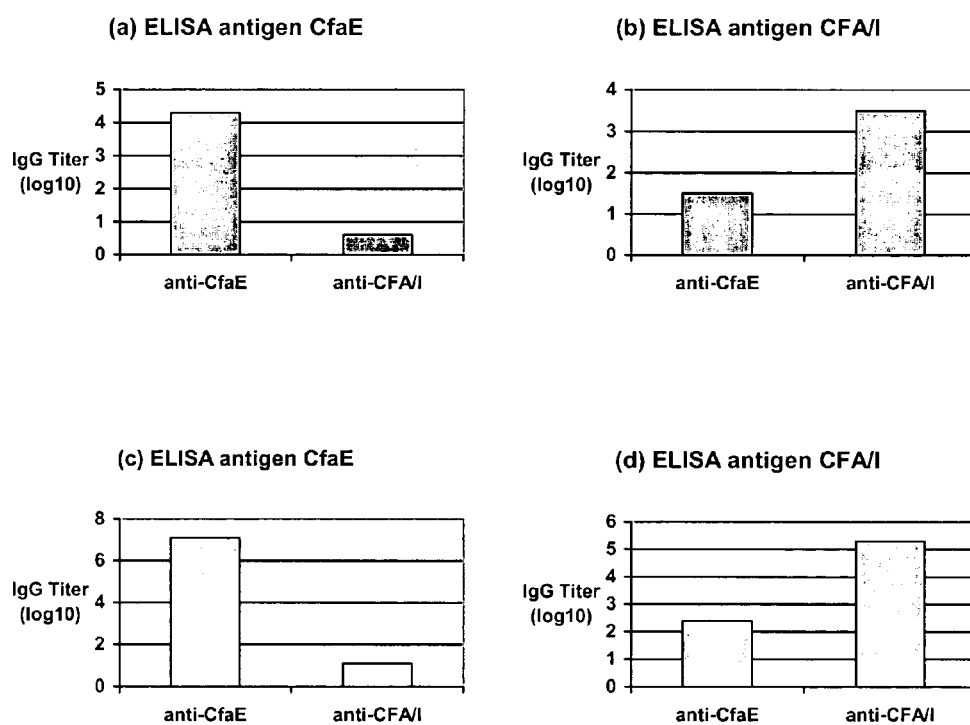


FIG 10

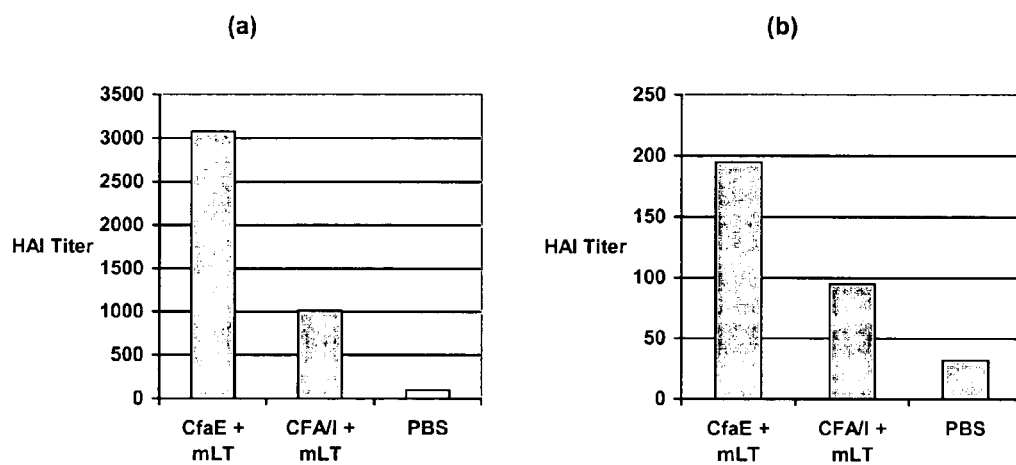


FIG 11

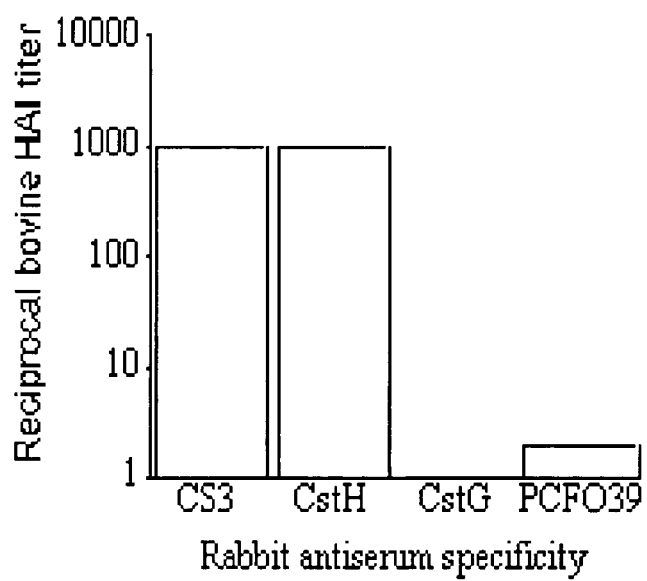


FIG 12

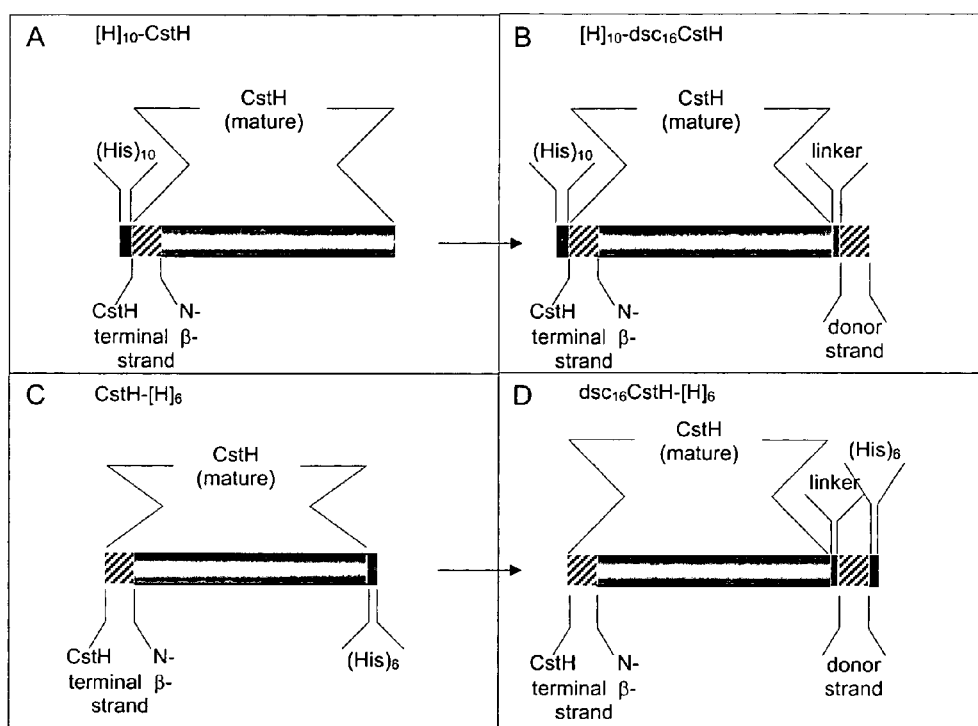


FIG 13

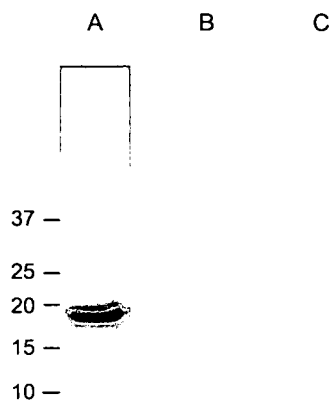


FIG 14

ADHESIN AS IMMUNOGEN AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI*

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional application 60/642,771 filed Jan. 11, 2005 the contents herein are incorporated by reference.

BACKGROUND OF INVENTION

1. Field of the Invention

The inventive subject matter relates to a method of inducing an immune response against diarrheagenic bacteria including enterotoxigenic *Escherichia coli* using bacterial fimbriae or fibrillar components. The method contemplates using *Escherichia coli* adhesins as immunogens against diarrheagenic bacteria.

2. Description of Related Art

Enterotoxigenic *Escherichia coli* (ETEC) are a principal cause of diarrhea in young children in resource-limited countries and also travelers to these areas (1, 2). ETEC produce disease by adherence to small intestinal epithelial cells and expression of a heat-labile (LT) and/or heat-stable (ST) enterotoxin (3). ETEC typically attach to host cells via filamentous bacterial surface structures known as colonization factors (CFs). More than 20 different CFs have been described, a minority of which have been unequivocally incriminated in pathogenesis (4).

Firm evidence for a pathogenic role exists for colonization factor antigen I (CFA/I), the first human-specific ETEC CF to be described. CFA/I is the archetype of a family of eight ETEC fimbriae that share genetic and biochemical features (5, 4, 6, 7). This family includes coli surface antigen 1 (CS1), CS2, CS4, CS14, CS17, CS19 and putative colonization factor O71 (PCFO71). The complete DNA sequences of the gene clusters encoding CFA/I, CS1 and CS2 have been published (8, 9, 10, 11, 12). The genes for the major subunit of two of the other related fimbriae have been reported (13, 6). The four-gene bioassembly operons of CFA/I, CS1, and CS2 are similarly organized, encoding (in order) a periplasmic chaperone, major fimbrial subunit, outer membrane usher protein, and minor fimbrial subunit. CFA/I assembly takes place through the alternate chaperone pathway, distinct from the classic chaperone-usher pathway of type I fimbrial formation and that of other filamentous structures such as type IV pili (14, 15). Based on the primary sequence of the major fimbrial subunit, CFA/I and related fimbriae have been grouped as class 5 fimbriae (16).

Similar, but distinct from class 5 fimbriae, coli surface antigen 3 (CS3) represents the common adhesive fibrilla of the ETEC colonization factor antigen II (CFA/II) complex. ETEC expressing these antigens are prevalent in many parts of the world. Although the conformational nature of CS3 containing fibrillae are even less understood than class 5 fimbriae, it is anticipated that these structures will also be important components in contemplated anti-ETEC vaccines.

Studies of CS1 have yielded details on the composition and functional features of Class 5 fimbriae (17). The CS1 fimbrial stalk consists of repeating CooA major subunits. The CooD minor subunit is allegedly localized to the fimbrial tip, comprises an extremely small proportion of the fimbrial mass, and is required for initiation of fimbrial formation (18). Contrary to earlier evidence suggesting that the major subunit mediates binding (19), recent findings have implicated the minor subunit as the adhesin and identified specific amino acid residues

required for in vitro adhesion of CS1 and CFA/I fimbriae (20). The inferred primary amino acid structure of those major subunits that have been sequenced share extensive similarity. Serologic cross-reactivity of native fimbriae is, however, limited, and the pattern of cross-reactivity correlates with phylogenetically defined subtaxons of the major subunits (13).

Implication of the minor subunits of Class 5 fimbriae as the actual adhesins entails scrutiny regarding the degree of their conservation relative to that of the major subunits. It was speculated that CooD and its homologs retained greater similarity due to functional constraints imposed by ligand binding requirements and/or its immunorecessiveness, itself attributable to the extremely large ratio of major to minor subunits in terms of fimbrial composition. Studies were conducted to examine the evolutionary relationships of the minor and major subunits of Class 5 ETEC fimbriae as well as the two assembly proteins (21). It was demonstrated that evolutionary distinctions exist between the Class 5 major and minor fimbrial subunits and that the minor subunits function as adhesins. These findings provide practical implications for vaccine-related research.

The nucleotide sequence of the gene clusters that encode CS4, CS14, CS17, CS19 and PCFO71 was determined from wild type diarrhea-associated isolates of ETEC that tested positive for each respective fimbriae by monoclonal antibody-based detection (21). The major subunit alleles of the newly sequenced CS4, CS14, CS17 and CS19 gene clusters each showed 99-100% nucleotide sequence identity with corresponding gene sequence(s) previously deposited in GenBank, with no more than four nucleotide differences per allele. Each locus had four open reading frames that encoded proteins with homology to the CFA/I class chaperones, major subunits, ushers and minor subunits. As previously reported (13), the one exception was for the CS14 gene cluster, which contained two tandem open reading frames downstream of the chaperone gene. Their predicted protein sequences share 94% amino acid identity with one another and are both homologous to other Class 5 fimbriae major subunits.

Examination of the inferred amino acid sequences of all the protein homologs involved in Class 5 fimbrial biogenesis reveals many basic similarities. Across genera, each set of homologs generally share similar physicochemical properties in terms of polypeptide length, mass, and theoretical isoelectric point. All of the involved proteins contain an amino-terminal signal peptide that facilitates translocation to the periplasm via the type II secretion pathway. None of the major subunit proteins contain any cysteine residues, while the number and location of six cysteine residues are conserved for all of the minor subunits except that of the *Y. pestis* homolog 3802, which contains only four of these six residues.

Type 1 and P fimbriae have been useful models in elucidating the genetic and structural details of fimbriae assembled by the classical chaperone-usher pathway (23, 24, 25). An outcome of this work has been development of the transformative principle of donor strand complementation, a process in which fimbrial subunits non-covalently interlock with adjoining subunits by iterative intersubunit sharing of a critical, missing β -strand (22, 26). Evidence has implicated this same mechanism in the folding and quaternary conformational integrity of *Haemophilus influenzae* hemagglutinating pili (27), and *Yersinia pestis* capsular protein, a non-fimbrial protein polymer (28). Both of these structures are distant Class I relatives of Type 1 and P fimbriae that are assembled by the classical chaperone-usher pathway. From an evolutionary perspective, this suggests that the mechanism of donor strand complementation arose in a primordial fimbrial system from which existing fimbriae of this Class have

derived. While donor strand complementation represents a clever biologic solution to the problem of protein folding for noncovalently linked, polymeric surface proteins, its exploitation by adhesive fimbriae other than those of the classical usher-chaperone pathway has not been demonstrated.

Common to fimbriae assembled by the alternate chaperone pathway and the classical chaperone-usher pathway are the requirement for a periplasmic chaperone to preclude subunit misfolding and an usher protein that choreographs polymerization at the outer membrane. That the fimbrial assembly and structural components of these distinct pathways share no sequence similarity indicates that they have arisen through convergent evolutionary paths. Nevertheless, computational analyses of the CFA/I structural subunits suggests the possibility that donor strand complementation may also govern chaperone-subunit and subunit-subunit interaction.

The eight ETEC Class 5 fimbriae clustered into three subclasses of three (CFA/I, CS4, and CS14), four (CS1, PCF071, CS17 and CS19), and one (CS2) member(s) (referred to as subclasses 5a, 5b, and 5c, respectively) (21). Previous reports demonstrated that ETEC bearing CFA/I, CS2, CS4, CS14 and CS19 manifest adherence to cultured Caco-2 cells (6, 22). However, conflicting data have been published regarding which of the component subunits of CFA/I and CS1 mediate adherence (19, 20).

This question of which fimbrial components is responsible for mediating adherence was approached by assessing the adherence-inhibition activity of antibodies to intact CFA/I fimbriae, CfaB (major subunit), and to non-overlapping amino-terminal (residues 23-211) and carboxy-terminal (residues 212-360) halves of CfaE (minor subunit) in two different in vitro adherence models (21). It was demonstrated that the most important domain for CFA/I adherence resides in the amino-terminal half of the adhesin CfaE (21).

The studies briefly described above provide evidence that the minor subunits of CFA/I and other Class 5 fimbriae are the receptor binding moiety (20). Consistent with these observations, because of the low levels of sequence divergence of the minor subunits observed within fimbrial subclasses 5a and 5b (20), the evolutionary relationships correlated with cross-reactivity of antibodies against the amino-terminal half of minor subunits representing each of these two subclasses (21).

An aspect of this invention is a method of inducing an immune response against ETEC strains incorporating either or both of class 5 fimbriae or conformationally stable fimbriae components responsible for fimbriae adhesion or CS3 fibrillae or conformationally stable CS3 fibrillae components.

SUMMARY OF THE INVENTION

Currently available vaccines against many diarrheagenic bacteria such as enterotoxigenic *Escherichia coli* are not adequately efficacious. New vaccine formulations against these organisms are critical, especially for developing countries where diarrheal diseases are most prevalent and medical infrastructure is limited.

An object of the invention is a method of inducing an immune response, including antibody responses, against class 5 *Escherichia coli* fimbriae by administration of polypeptides encoding fimbrial adhesin or fibrillar adhesion.

A still further object is the prevention of colonization of *Escherichia coli* by inhibiting adherence of fimbriae or fibrillae to host cells.

An additional object is the construction of conformationally-stable and protease resistant adhesin polypeptide constructs for use in vaccine formulations.

A still additional object is the use of the adhesin polypeptide constructs to induce immunity to *Escherichia coli*, including enterotoxigenic *E. coli*, fimbriae.

These and other objects of the invention are accomplished by employing *Escherichia coli* adhesin polypeptides as an immunogenic component to induce immunity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Inhibitory effects of different F_{ab} antibody preparations on adherence of strain H10407 (CFA/I) in two in vitro adherence models.

FIG. 2. Median reciprocal bovine hemagglutination inhibition (HAI) titers (plotted on \log_2 scale) of F_{ab} antibody preparations against whole fimbriae or the amino-terminal domain of the minor fimbrial subunit of CFA/I (Panel A), and CS17 (Panel B), for ETEC type strains expressing the colonization factor indicated along the x-axis. Results represent the median of at least 4 experiments, each performed in duplicate. P values are for the differences in HAI titers between the whole fimbriae and minor subunit antibody preparations.

FIG. 3. Inhibitory effects of F_{ab} antibodies against intact fimbriae and the N-terminal half of the minor subunit of CFA/I (open bar graphs) and CS17 (black bar graph) in Caco-2 cell adherence assays with ETEC bearing homologous (CFA/I only, upper left panel) and heterologous fimbriae.

FIG. 4. A highly conserved β -strand motif in the major structural subunits of Class 5 fimbriae. This is a multiple alignment of the amino-termini of the mature form of the major subunits, with consensus sequence shown below. This span is predicted to form an interrupted β -strand motif spanning residues 5-19 (demarcated by yellow arrows below consensus). Shading of conserved residues indicate class as follows: blue, hydrophobic; red, negatively charged residues; turquoise, positively charged residues; and green, proline. Abbreviations: Bcep, *Burkholderia cepacia*; Styp, *Salmonella typhi*. U, hydrophobic residue; x, any residue; Z, E or Q.

FIG. 5. Schematic diagrams of CfaE construct.

FIG. 6. Elution profile of dsc₁₉CfaE(His)₆ upon gel filtration with Superdex 75 (16/60) in 20 mM MES and 100 mM NaCl.

FIG. 7. Inhibitory effects of anti-CFA/I and anti-dsc₁₉CfaE [His]₆ antiserum on mannose-resistant hemagglutination (MRHA) of CFA/I-ETEC (prototype strain H10407; LTST, CFAI, O78:H11) and ETEC that express related subclass 5a fimbriae CS4 (strain WS2560B; LTST, CS4+CS6, O25:H-) and CS14 (strain WS3294A; ST, CS14, O78:H18).

FIG. 8. Purified dsc₁₉CfaE(His)₆ in particulate form induces mannose-resistant hemagglutination (MRHA) of human type A and bovine erythrocytes.

FIG. 9. Antibody induction following orogastric or intranasal administration in mice of dscCfaE plus mLT or CFA/I plus mLT.

FIG. 10. Anti-CfaE and anti-CFA/I ELISA binding activity by ELISA using either dscCfaE or CFA/I as antigen.

FIG. 11. HAI activity of serum from mice immunized with dscCfaE plus mLT or CFA/I plus mLT.

FIG. 12. Hemagglutination inhibition of rabbit polyclonal antiserum generated against native CS3, purified CstH, CstG and PCF039 fimbriae.

FIG. 13. Schematic representation of components of CstH construct. Panel A, illustrates mature CstH of CS3 with histidine tag attached at its N-terminal end. Panel B illustrates the construct in panel A but with a short linker polypeptide attached at the C-terminal end of the mature CstH construct which in-turn has a duplicated 16 amino acid CstH N-terminal region attached at its C-terminus. Panel C illustrates the construct of Panel A but with a (His)₆ tag inserted at the C-terminus, verses at the N-terminus. Panel D illustrates a similar construct as in Panel B but with a smaller (His)₆ on the C-terminus of the duplicated CstH region donor strand verses a (His)₁₀ at the N-terminus.

FIG. 14. SDS PAGE and western blot analysis of purified dsc₁₆CstH[His]₆.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention relates to methods and a biological composition for the induction of anti-adhesive immune responses by the administration of fimbriae or fimbrial adhesin components. I hereby state that the information recorded in computer readable form is identical to the written sequence listing.

Adhesin, the distal molecular component of enterotoxigenic *Escherichia coli* fimbriae, are the likely effectors for bacterial attachment to host cells (21). Therefore, adhesins are critical for bacterial colonization and pathogenicity.

The inventive method, immunization with adhesive subunits of class 5 fimbriae, will induce principally immunoglobulin mediated immunity, that specifically binds to bacterial adhesin to disrupt colonization of diarrheagenic bacteria. The method, therefore, will provide superior and more efficacious immunity against diarrheagenic bacteria. Furthermore, the use of fimbrial adhesin subunits in place of intact fimbriae or whole bacteria will likely require significantly less antigen to elicit immunity with improved efficacy of immunity.

The invention provides a method for inducing immunity by administration of polypeptides encoding *Escherichia coli* adhesin, which is the host-cell adhesive component structurally located at the tip of *Escherichia coli* fimbriae. The archetype fimbriae, colonization factor antigen I (CFA/I) is found on the most important enterotoxigenic *Escherichia coli* (ETEC) strains. However, because of the close evolutionary relationship of the ETEC adhesins, other class 5 fimbriae can also be utilized.

Conformational stability, and potentially protease resistance, of adhesin polypeptides is important to ensure maximum immunogenicity. Conformational integrity of adhesin monomers is conferred by a donated β -strand provided by an adjacent major structural fimbrial monomer. For example, conformational stability of the CFA/I adhesin, CfaE, is provided by the donor β -strand from CfaB (SEQ ID NO 24).

For improved anti-fimbrial adhesin immunity, an aspect of the invention is conferral of conformational stability on adhesin polypeptide sequences. In order to ensure conformational stability of adhesin polypeptide immunogens with concomitant improved efficaciousness of vaccines, an aspect of this invention is polypeptide constructs designed to operatively provide a donor β -strand to adjacent adhesin polypeptide sequences. The constructs are composed of adhesin polypeptides linked at the C-terminal end to a linker polypeptide which is in turn linked, at the C-terminal end, to a polypeptide encoding all or a portion of a major fimbrial structural subunit, such as CfaB.

EXAMPLE 1

Adhesin is the Most Important Vaccine-Related Enterotoxigenic *Escherichia coli* Bacterial Component

Class 5 *Escherichia coli* Fimbriae Binding

CFA/I is the archetype of a family of ETEC fimbriae sharing genetic and biochemical features (5, 4, 6, 7). The gene operons are composed of a periplasmic chaperone, major fimbrial subunit, outer membrane usher protein and a minor fimbrial subunit. Based on the major subunit sequence, CFA/I and related fimbriae have been grouped together as class 5

fimbriae (16, 21). Studies have confirmed that there is a confirmed functional distinction between class 5 major and minor fimbrial subunits and that the minor subunits serve as adhesins. Therefore, the minor subunits are the most important component of fimbriae for vaccine construction.

Type strains that individually express each of the Class 5 ETEC fimbriae were characterized with respect to erythrocyte adherence by mannose-resistant hemagglutination (MRHA) with type A human, bovine, and chicken erythrocytes (21). The phenotypes of all ETEC strains used in adhesion experiments are shown in Table 1. The type strains that expressed CS1, CS4, CS14, CS17, CS19 and PCFO71 were each isolated from the feces of young children with diarrhea, as part of a longitudinal study of childhood diarrhea in Egypt (29).

ETEC strains were tested for mannose-resistant hemagglutination (MRHA) of human type A, bovine, and chicken erythrocytes. MRHA methods were based on previously described procedures (30). The results shown are shown in Table 1.

In these studies, for routine propagation and protein expression, bacteria were grown in Luria-Bertani medium (31) or in rich medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 2 g glucose per L). For hemagglutination and tissue culture adherence assays, cultures were grown on CFA agar (32) with or without addition of 1.5 g of Bacto Bile Salts no. 3 (Difco, Detroit, Mich.) per liter. Ampicillin (62.5 μ g/ml) and kanamycin (50 μ g/ml) were added as needed for selection pressure. Human erythrocytes were harvested as needed from a single volunteer donor, and bovine and chicken erythrocytes were purchased from Lampire Laboratories (Pipersville, Pa.). Erythrocytes were stored for up to two weeks at 4° C. in Alsever's solution prior to use. Just before each assay, erythrocytes were washed and suspended in PBS with 0.5% D-mannose to a final concentration of 3%. Bacteria were grown overnight at 37° C. and suspended in PBS with 0.5% D-mannose to a final concentration of about 1×10^{10} colony forming units (cfu)/ml. Equal volumes (25 μ l each) of 3% red cells, bacterial suspension, and PBS with 0.5% D-mannose were added and mixed in wells on a 12-well ceramic tile (CoorsTec, Golden, Colo.), rocked on ice for 20 min, graded by visual inspection, and scored as follows: negative, indicating no MRHA activity; 1+ indicating a low, weak reaction; 2+ denoting a moderate reaction; 3+ indicating a strong reaction; and 4+ a nearly instantaneous and complete reaction involving all of the erythrocytes.

We also analyzed component subunit adherence to Caco-2 cells. The results of these studies are also shown in Table 1. Adherence assays were performed as described previously (33, 34) with minor modifications. Briefly, Caco-2 cells were maintained at 37° C. in air supplemented with 5% CO₂ in EMEM media (Minimum Essential Medium, Eagle's, in Earle's Balanced Salt Solution) supplemented with 2 mM L-glutamine, 20% fetal bovine serum, 0.1 M non-essential amino acids, 1 mM sodium pyruvate, and 1.5 g/liter sodium bicarbonate. Cells were seeded in 24 well plates (Costar, Corning, N.Y.) loaded with tissue culture-treated glass cover slips (Fisher Scientific), and incubated for 14 days (\pm 1 d) to post-confluence, washed with PBS, and covered with 750 μ l of the supplemented EMEM prior to the assay. Bacterial strains were grown on CFA agar with or without bile salts overnight at 37° C. and suspended to 1×10^9 bacteria/ml in supplemented EMEM with 1% D-mannose. The suspension was added to the tissue culture wells at a final concentration of 2.5×10^8 bacteria/ml. Plates were incubated, washed, fixed, stained and mounted as described (34), and observed microscopically. The number of bacteria adherent to 100 randomly

selected cells was counted to give an average number of cells with at least 1 adherent bacteria (adherence index 1), and number of bacteria per Caco-2 cell with at least one adherent bacteria (adherence index 2). For each bacterial strain, a minimum of 3 experiments was done in duplicate to determine the adherence indices, expressed as the mean \pm standard deviation (SD).

It has previously been reported that ETEC bearing CFA/I, CS2, CS4, and CS14 and CS19 manifest adherence to cultured Caco-2 cells (6, 22). Caco-2 cell adherence assays on each of the ETEC type strains bearing the Class 5 fimbriae were performed to confirm these findings and quantify the level of adherence for each strain. The results (Table 1) indicated that indeed the strains bearing CFA/I, CS4, CS14 and CS2 each showed moderate to high level Caco-2 cell adherence, while a lower level of adherence was observed for the CS19-bearing strain. In contrast, the strains expressing CS1, CS17 and PCFO71 manifest marginal levels of adherence. Transformation of the strains bearing Subclass 5b fimbriae with a plasmid containing the CFA/I positive regulator *cfaD* was associated with an increase in Caco-2 cell adherence only for the CS19-ETEC strain WS0115A.

Considering the evolutionary relationships of the Class 5 ETEC fimbriae, it can be seen that there are some distinguishing functional characteristics that correlate with their phylogeny. Subclass 5a fimbriae are distinct from the others by virtue of their ability to cause MRHA of human type A erythrocytes. With the exception of the CS19-ETEC, Subclass 5b fimbriae show weak if any adherence to cultured Caco-2 cells, differentiating them from the other two subclasses.

TABLE 1

In vitro adherence phenotypes of ETEC type strains bearing CFA/I and related Class 5 fimbriae.						
Strain	CF type	MRHA			Caco-2 cell adherence ^a	
		humanA	bovine	chicken	Index 1 ^b	Index 2 ^c
H10407	CFA/I	4+	4+	3+	54.3 \pm 15.4	14.2 \pm 2.7
WS2560B	CS4	2+	2+	1+	26.7 \pm 7.0	2.9 \pm 1.6
WS3294A	CS14	2+	3+	3+	63.3 \pm 5.8	8.2 \pm 2.4
WS1974A	CS1	—	3+	—	12.7 \pm 8.6	2.1 \pm 1.1
WS2173A	PCFO71	—	4+	2+	12.7 \pm 6.2	1.8 \pm 0.6
WS6788A	CS17	—	4+	—	10.0 \pm 2.6	1.1 \pm 0.2
WS0115A	CS19	—	4+	2+	19.3 \pm 6.0	1.8 \pm 0.8
C91f	CS2	—	3+	3+	69.3 \pm 4.7	15.1 \pm 4.7

^aRepresents the mean of at least 3 experiments, each done in duplicate.

^bMean proportion of Caco-2 cells with at least one adherent bacteria (\pm SD)

^cMean number of adherent bacteria per Caco-2 cell with at least one adherent bacteria (\pm SD)

Adhesin are Responsible for Fimbriae Binding.

In order to determine the fimbriae components responsible for host cell binding the ability of specific antibody to adhesins to inhibit CFA/I and CS1 fimbriae adherence was analyzed (21). We further evaluated the question whether antibody to these moieties would cross-react in accordance to evolutionary relationships. This was evaluated indirectly by measuring adhesion-inhibition activity of antibodies to intact CFA/I fimbriae, CfaB (major subunit), and to non-overlapping amino-terminal (residues 23-211) and carboxy-terminal (residues 212-360) halves of CfaE (minor subunit) in two different in vitro adherence models (see SEQ ID No. 4 for sequence of CfaE).

CFA/I and CS17 fimbriae were purified as previously described (35, 36). Rabbit polyclonal antibody preparations were prepared against MBP-CfaB₂₄₋₁₇₀, MBP-CfaE₂₃₋₂₁₁, MBP-CfaE₂₁₂₋₃₆₀, MBP-CsbD₁₉₋₂₁₄, and against native CFA/I and CS17 fimbriae (21). Each of these above *E. coli*

type strains described above, except for those that expressed CFA/I, CS1 and CS2, was also the source of DNA for sequence analysis of the corresponding fimbrial operon. *E. coli* BL21 (F⁻ ompT hsdSB(rB⁻mB⁻) gal dcm) was obtained from a commercial source (New England Biolabs, Beverly, Mass.) and used for cloning and expression of maltose-binding protein (MBP) fusions. Rabbit immunizations and antiserum collection were performed by Harlan Bioproducts for Science, Inc. (Indianapolis, Ind.). Purified IgG was derived from each antiserum using Hi-Trap Protein G columns as directed by the manufacturer (Amersham Pharmacia, Piscataway, N.J.). From each of these preparations, F_{ab} fragments were generated using the Pierce ImmunoPure F_{ab} preparation kit (Pierce, Rockford, Ill.).

ETEC strains were tested for mannose-resistant hemagglutination (MRHA). For hemagglutination inhibition (HAI) assays, each bacterial strain was used at a concentration corresponding to two times the minimal hemagglutination titer (2 \times MHT). The MHT was determined at the start of each HAI assay day by making serial two-fold dilutions of the bacterial suspension (from a starting concentration of 1 \times 10¹⁰ cfu/ml) in PBS. A total of 25 μ l of each dilution was added to equal volumes of 3% erythrocyte suspension and PBS with 0.5% D-mannose and rocked on ice. The MHT was defined as the reciprocal of the lowest concentration of bacteria showing at least 1+MRHA. To determine the HAI titer of each F_{ab} antibody preparation, a two-fold dilution series was made starting with the stock antibody solution (2 mg/ml). A 25 μ l volume of each F_{ab} dilution was added to an equal volume of a 2 \times MHT bacterial suspension in the ceramic tile wells and pre-incu-

bated at room temperature with rocking for 20 min. An equal volume of erythrocyte suspension (3%) was then added to each well, the tiles were rocked on ice for 20 min, and MRHA was scored as above. The HAI titer was expressed as the reciprocal of the highest dilution of antiserum that completely inhibited MRHA.

For Caco-2 cell adherence inhibition experiments, a 120 μ l aliquot of F_{ab} antibody preparation (2 mg/ml starting concentration) was added to 480 μ l of the bacterial suspension and pre-incubated at room temperature for 20 min. Addition of PBS in place of the antibody preparation served as a negative control in each experiment. A 250 μ l aliquot of the bacteria/antibody mixture (2.5 \times 10⁸ bacteria/ml) was then added to tissue culture wells. The cells were incubated, processed, and analyzed as described above. The level of inhibition was determined by comparing the primary adherence index with and without addition of antibody. For each test bacteria/antibody preparation, a minimum of 3 experiments was per-

formed in duplicate. In the Caco-2 adherence studies, adherence conducted in the presence of each antibody preparation was compared to that with addition of PBS, using a one-tailed Student T test, assuming unequal variance between samples. For HAI experiments, reciprocal titers between experimental groups were compared using the Wilcoxon signed rank test for paired samples (one-tailed) using XLSTAT data analysis software.

Each of four antibody preparations was assessed for ability to inhibit the adherence of strain H10407 (CFA/I) in MRHA and Caco-2 cell adherence assays. FIG. 1 (A) shows median reciprocal hemagglutination inhibition (HAI) titers of F_{ab} antibodies specific for MBP, CFA/I, CfaB, CfaE₂₃₋₂₁₁ (denoted as CfaE_N), and CfaE₂₁₂₋₃₆₀ (denoted as CfaE_C), plotted on log₂ scale. Values below a reciprocal of 2 (limit of detection) were arbitrarily plotted as 1.05 for graphing purposes. FIG. 1 (B) shows mean Caco-2 cell adherence index (% Caco-2 cells with at least 1 adherent bacterium, \pm SD) of H10407 after pre-incubation of bacteria with F_{ab} antibodies with the same specificities. All preparations were tested in at least three experiments, each done in duplicate.

The highest human A erythrocyte hemagglutination inhibition (HAI) activity was observed with F_{ab} specific for CfaE₂₃₋₂₁₁, while CfaB antibodies manifest a much lower level of HAI activity (FIG. 1 (A)). No HAI activity was detectable with F_{ab} antibodies against CFA/I or CfaE₂₁₂₋₃₆₀. Consistent findings were observed in Caco-2 cell adherence inhibition assays, in that the highest inhibitory activity was attributable to anti-CfaE₂₃₋₂₁₁ F_{ab} fractions (FIG. 1(B)). In this assay anti-CFA/I F_{ab} antibodies showed a lower level of inhibition, and preparations specific for CfaB and CfaE₂₁₂₋₃₆₀ showed no detectable effect. Taken together, these findings suggest that the most important domain for CFA/I adherence resides in the amino-terminal half of CfaE.

To test the hypothesis that evolutionary relationships would correlate with cross-reactivity of antibodies against the amino-terminal half of minor subunits representing the 5a and 5b subclasses the inhibitory effect of anti-CfaE₂₃₋₂₁₁ F_{ab} on adherence of wild type strains expressing heterologous Class 5 fimbriae was assessed. Consistent with our predictions, anti-CfaE₂₃₋₂₁₁ inhibited bovine MRHA of CS4-ETEC and CS14-ETEC (FIG. 2 (A)). In comparison, anti-CFA/I F_{ab} antibodies inhibited bovine MRHA of CFA/I-ETEC to a lesser degree than the anti-CfaE₂₃₋₂₁₁ while failing to inhibit MRHA of ETEC bearing CS4 or CS14. Identical results were obtained using human erythrocytes, except that anti-CFA/I F_{ab} failed to display CFA/I-ETEC HAI. Neither antibody preparation inhibited bovine MRHA of ETEC bearing heterologous CFs of the other two subclasses.

These findings were corroborated by measuring the inhibitory effects of each F_{ab} preparation in the Caco-2 cell adherence assay. Anti-CfaE₂₃₋₂₁₁ antibodies inhibited the adherence of CS4-ETEC and CS14-ETEC when compared to the adherence level when bacteria were pre-incubated with PBS (FIG. 3) or with anti-MBP antibodies (data not shown). The diminished adherence of CS14-ETEC did not, however, achieve statistical significance. At the same concentration, anti-CFA/I antibodies inhibited Caco-2 cell adherence of H10407 (CFA/I), though to a significantly lesser degree than did anti-CfaE₂₃₋₂₁₁ F_{ab} . Anti-CFA/I F_{ab} did not, however, inhibit binding of ETEC bearing heterologous CFs of the same (FIG. 3) or different subclasses (data not shown).

To strengthen these findings further, we produced antibodies to the amino-terminal half of the CS17 (Subclass 5b) minor subunit CsbD and assessed its inhibitory activity along with that of anti-CS17 fimbrial antibodies in the MRHA and Caco-2 tissue culture cell model systems. Both anti-CS17 and

anti-CsbD₁₉₋₂₁₄ F_{ab} antibodies exhibited bovine erythrocyte HAI activity for ETEC bearing CS17, with the HAI titer of anti-CsbD₁₉₋₂₁₄ being significantly higher (FIG. 2B). Distinct from the anti-CS17 F_{ab} antibodies, the anti-CsbD₁₉₋₂₁₄ F_{ab} fraction also manifest significant HAI activity for ETEC bearing each of the other Subclass 5b fimbriae. Notably, the intrasubclass CF-heterologous HAI activity of anti-CsbD₁₉₋₂₁₄ antibodies was closer in magnitude to its CS17-ETEC HAI activity than for the comparable effects of anti-CfaE₂₃₋₂₁₁ antibodies. This finding was anticipated given the higher degree of identity of the minor subunits within Subclass 5b. Neither preparation inhibited bovine MRHA of ETEC bearing CFs of the other two subclasses.

In the Caco-2 cell adherence assay, we assessed the inhibitory effects of the same antibody preparations for CS19-ETEC, the only Subclass 5b fimbriae that appears to specifically adhere to Caco-2 cells. Here too we found that anti-CsbD₁₉₋₂₁₄ but not anti-CS17 antibodies showed significant inhibition of CS19-ETEC adherence (FIG. 3). In FIG. 3, the strain used in experiments is shown above each graph. The y-axes indicate the Caco-2 cell adherence index (percentage of Caco-2 cells with at least one adherent bacteria). Results represent the mean (\pm SD) of at least 3 experiments, each performed in duplicate. P values are for the differences between the negative control (PBS) and the indicated antibody preparation. Neither preparation inhibited Caco-2 cell adherence of ETEC expressing representative Subclass 5a or 5c fimbriae (data not shown).

EXAMPLE 2

Conformationally Stable Donor-Strand Complemented Class 5 Adhesive Fimbriae-adhesin Immungenic Construct

Computational analyses of the CFA/I structural subunits suggests that donor strand complementation governs chaperone-subunit and subunit-subunit interaction. Therefore, we constructed a conformationally-stable construct wherein an amino-terminal donor β -strand of CfaB provides an in cis carboxy-terminal extension of CfaE to confer conformational stability and protease resistance to this molecule forming a soluble monomer capable of binding human erythrocytes.

We generated a multiple alignment of the amino acid sequences of the eight homologs of the major and minor subunits of Class 5 ETEC fimbriae to identify common structural motifs. Secondary structure prediction algorithms indicated that both subunits form an amphipathic structure rich in β -strands distributed along their length. Twenty six percent of the consensus minor subunit sequence is predicted to fold into a β -conformation, comprising 17 interspersed β strands, which might be expected to form a hydrophobic core. In Cis Donor Strand Complementation of cfaE.

Two highly conserved structural motifs were identified, one of which is shared between the carboxyl termini of major and minor subunits alike and another found at the amino-terminal end of the mature (post-signal peptide cleavage) form of the major subunits. Multiple alignment of the major and minor subunits together revealed a common motif at the carboxyl terminus of each protein representing the sequence motif AGxYxGxUxUxUT(x)₃₋₆-COOH, where U represents any hydrophobic residue and x represents a residue of unspecified nature (FIG. 4). Sakellaris et al have previously suggested that this span denotes a β -zipper motif, analogous to that of Class I fimbrial subunits that may play a role in fimbrial subunit-chaperone interaction (37).

The major subunits of Class 5 fimbriae share a very highly conserved amino-terminal span predicted to form a β strand (FIG. 4), differing in this respect from the minor subunits. Based on its predicted structure and location, this span serves as a β -strand-like structure that is donated to neighboring CfaB subunits along the alpha-helical stalk and to CfaE at the fimbrial tip. For sequences serving as CfaB major subunit donor strand see SEQ ID No. 7. For donor strands for other adhesin monomers see SEQ ID No. 8-15.

The highly conserved nature of the amino-terminal β strand of CfaB and its homologs, together with the precedent that the amino-terminus of type 1 fimbrial subunits functions as the exchanged donor strand in filament assembly suggested this as a good candidate for the donor β strand that noncovalently interlocks CFA/I subunits. To test this hypothesis with respect to the minor adhesive subunit, we engineered a plasmid to express a CfaE variant containing a C-terminal extension consisting of a flexible hairpin linker (DNKQ (SEQ ID No. 1) followed by the first 13 amino acid residues of mature CfaB (FIG. 5). FIG. 5(A) illustrates, schematically, the domains of independent CfaE variant constructs with C-terminal extensions comprising the N-terminal β -strand span of CfaB varying in length from 10 to 19 residues. Each construct contains a short flexible linker peptide (DNKQ) intercalated between the C-terminus of the native CfaE sequence and the donor β -strand. The vertical arrow identifies the donor strand valine that was modified to either a proline (V7P) to disrupt the secondary β -strand motif. FIG. 5(B) shows a western blot analysis of periplasmic concentrates from the series of strains engineered to express CfaE and the variants complemented in cis with varying lengths of the amino-terminal span of mature CfaB. The primary antibody preparations used were polyclonal rabbit antibody against CfaE. Lanes correspond to preparations from the following constructs: Lane 1, dsc₁₀CfaE; 2, dsc₁₁CfaE; 3, dsc₁₂CfaE; 4, dsc₁₃CfaE; 5, dsc₁₃CfaE[V7P]; 6, dsc₁₄CfaE; 7, dsc₁₆CfaE; 8, dsc₁₉CfaE; and 9, CfaE. Molecular weight markers (in kD) are shown to the left. FIG. 5(C) is a schematic representation of the engineered components of dsc19CfaE(His)₆, containing the native CfaE sequence (including its Sec-dependent N-terminal signal sequence), with an extension at its C-terminus consisting of a short linker sequence (i.e., DNKQ), the 19 residue donor strand from the N-terminus of mature CfaB, and a terminal hexahistidine affinity tag.

PCR products of cfaE were inserted into plasmid vectors by in vitro recombination using the Gateway® system (Invitrogen, Carlsbad, Calif.). Primers with the following sequences were used for the initial cloning into pDONR207™: dsc-CfaE 13-1 (forward), 5'-TCG ACA ATA AAC AAG TAG AGA AAA ATA TTA CTG TAA CAG CTA GTG TTG ATC CTT AGC-3' (SEQ ID No. 16); and dsc-CfaE 13-2 (reverse), 5'-TCG AGC TAA GGA TCA A C A C T A G C T GTT ACA GTA ATA TTT TTC TCT ACT TGT TTA TTG-3' (SEQ ID No 17). The PCR products flanked by attB recombination sites were cloned into the donor vector pDONR201™ (Gateway® Technology, Invitrogen, Carlsbad, Calif.), using the Gateway BP® reaction to generate the entry vector pRA13.3. In the Gateway LR® reaction the gene sequence was further subcloned from pRA13.3 into the modified expression vector pDEST14-Kn^r (vector for native expression from a T7 promoter) to generate the plasmid pRA14.2. The pDEST14-Kn^r vector was constructed by modifying pDEST14® (Gateway® Technology, Invitrogen, Carlsbad, Calif.) by replacement of ampicillin with kanamycin resistance. The presence of the correct cfaE was confirmed by sequence analysis. *E. coli* strain BL21ST™ (Invitrogen, Carlsbad, Calif.) was used for the expression of the

pRA14.1 and related CfaE donor strand complemented constructs. Cultures were grown overnight at 30° C. in LB medium without NaCl (LBON) containing 50 µg/ml kanamycin. An aliquot of the overnight culture was diluted 1:50 in LBON medium and grown at 30° C. At OD₆₀₀ of 0.5, NaCl was added to a final concentration of 200 mM, and the cells were grown at 30° C. for 3 hours. The induced cells were harvested, washed, and collected by centrifugation. Induction of protein expression was achieved by the addition of NaCl, followed by fractionation and analysis of periplasmic contents to determine the relative recovery of each protein.

We found that little CfaE was recoverable from the parent strain that expressed native CfaE, while the dsc₁₃CfaE construct yielded an obvious band on western blot analysis of the periplasmic fraction (FIG. 5 (B)). To confirm that the improved stability was specifically related to the β strand motif of the C-terminal extension, we made site-specific mutations in the central valine, changing it to either of two residues expected to break the β strand. The resultant constructs, dsc₁₃CfaE[V7P] and dsc₁₃CfaE[V7S] yielded little recoverable protein suggesting that the β strand is important to the observed stability achieved by the 13 amino acid C-terminal extension (FIG. 5 (B)).

We then established whether a donor strand length restriction exists for stabilization of CfaE. A series of plasmids were constructed to express variants of CfaE in the same general format but with the added CfaB N-terminal β -strand varying from the first ten to as many as 19 amino acids. As shown in FIG. 5B, a donor strand length of at least the first 12 amino acids was required to achieve measurable recovery of CfaE. At the upper end of strand length, we found that as many as 19 amino acids provided the necessary information to achieve recovery of CfaE.

Chaperone-Adhesin Complex Formation and in Cis Donor Strand Complementation.

CooD (SEQ ID 33), the CS1 homolog of CfaE, has been shown to form a periplasmic complex with its cognate chaperone CooB as well as with the CooA major fimbrial subunit. Analogous to type 1 fimbrial subunits, it is possible that a discrete hydrophobic groove of CooD and CfaE noncovalently interact with their respective chaperones in the process of biogenesis by the mechanism of donor strand complementation and exchange. To test such a model, we co-expressed a C-terminal hexahistidine-tagged variant of CfaA either with native CfaE or with dsc₁₉CfaE and looked for the formation of bimolecular chaperone-adhesin complexes. When native CfaE was co-expressed with CfaA (His)₆, the two proteins co-purified upon nickel affinity chromatography, indicating the formation of a complex. In contrast, co-expression of dsc₁₉CfaE with CfaA(His)₆ followed by affinity chromatography yielded only CfaA(His)₆. This suggests that the C-terminal β strand contributed by CfaB in cis precludes chaperone-adhesin complex formation. Purification and Characterization of dsc₁₉CfaE(His)₆.

Densitometric analyses of western blots of the various dscCfaE constructs containing 13 to 19 CfaB residues revealed little difference in recovery to suggest one variant over another in terms of superior fit. To ensure that we were working with a CfaE variant with as much of its hydrophobic cleft covered as possible, we selected dsc₁₉CfaE for purification and characterization. To facilitate purification, we added a hexahistidine tag to the carboxyl-terminus to yield dsc₁₉CfaE(His)₆, as schematically shown in FIG. 5 (C).

In FIG. 6, a chromatographic analysis shows elution volume of dsc₁₉CfaE(His)₆ (arrow), as well as molecular weight controls that include (A) albumin, 67,000 D; (B) ovalbumin, 43,000 D; (C) chymotrypsinogen A, 25,000 D; and (D) ribo-

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nuclease A, 13,700 D. Controls were separated in two different runs (B and D; and A and C), as was dsc₁₉CfaE(His)₆, and the three chromatograms were superimposed. The inset shows the calibration curve of derived from the 4 molecular weight standards, each of which runs as a monomer. The molecular weight of dsc₁₉CfaE(His)₆ was determined to be 38,961 D (see drop-down dotted line) using the formula $K_{av} = -0.1437 \ln(MW) + 1.6973$, where the slope and intercept were derived from the line through the standards generated by a logarithmic fit ($R^2 = 0.977$). This matches closely with the calculated mass of mature dsc₁₉CfaE(His)₆ (M_r , 40940).

A two-step chromatographic purification process was developed and refined using nickel affinity followed by cation exchange, which yielded soluble dsc₁₉CfaE(His)₆ of ca. 94% purity (FIG. 6). The results of N-terminal sequence analysis (DKNPGSENMTNTIGPHDRGG) (see SEQ ID No. 18) confirmed the identity of dsc₁₉CfaE(His)₆ and also validated accuracy of the signal peptide cleavage site prediction method of von Heijne (38). On gel filtration, mature dsc₁₉CfaE(His)₆ showed an elution profile consistent with a size of 40,869 daltons, indicating that dsc₁₉CfaE(His)₆ exists in a monomeric state.

Published evidence has indirectly implicated CfaE as the adhesive component of CFA/I fimbriae (20, 21). To directly test this premise, we adsorbed dsc₁₉CfaE(His)₆ onto 3 μ m latex beads and tested the hemagglutination properties of these particles in the presence of mannose by MRHA (FIG. 7). In FIG. 7, the upper graph shows HAI titers of the two antisera with bovine erythrocytes and the lower panel with human type A erythrocytes. Results represent the median of at least 5 experiments, each performed in duplicate. Neither antiserum manifest HAI activity when pre-incubated with prototype ETEC that express other class 5 fimbriae of the other two subclasses. Beads coated with dsc₁₉CfaE(His)₆ induced MRHA of human and bovine erythrocytes. In contrast, beads coated with purified CfaB (major subunit) did not induce MRHA of human bovine or chicken erythrocytes.

To corroborate the specificity of dsc₁₉CfaE(His)₆ hemagglutination effect, we determined the hemagglutination inhibition (HAI) titer of rabbit polyclonal anti-dsc₁₉CfaE(His)₆ serum against wild type CFA/I-ETEC (FIG. 8). In FIG. 8, each purified protein preparation was adsorbed to 3- μ m polystyrene beads, blocked with glycine, and added to 3% (vol/vol) suspension of fresh human type A (Row 1), bovine (Row 2) and chicken erythrocytes (Row 3) in porcelain tile wells. MRHA was visually determined after 20 minutes of rocking on ice. Column 2 shows human and bovine MRHA positive phenotype of dsc₁₉CfaE(His)₆ and Column 3 shows the corresponding negative MRHA phenotypes of the CFA/I major subunit dsc₁₉CfaB(His)₆, CFA/I native fimbriae (Column 1) and the CFA/I periplasmic chaperone protein CfaA(His)₆ (Column 4) served as positive and negative controls, respectively.

As shown in FIG. 8, the anti-dsc₁₉CfaE(His)₆ serum exhibited a median HAI titer of 1:12,288, six-fold greater but not statistically different than the median HAI titer of anti-CFA/I serum. Anti-dsc₁₉CfaE(His)₆ serum also registered HAI titers exceeding those of CFA/I antiserum against bacteria that expressed CS4 and CS14, the two Class 5 fimbriae of the same subclass as CFA/I (FIG. 8). Neither of these antisera revealed detectable HAI titers against bacteria that express fimbriae of the two other defined Class 5 subgroups. Ultrastructural Localization of CfaE in CFA/I Fimbriae.

It was previously suggested that CfaE localizes to the distal tip of CFA/I fimbriae based on inference from genetic manipulations and crude bacterial surface fractionation studies (34). However, the imprecision of these approaches has

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left the question of CfaE localization open to debate. Using high-titer polyclonal antiserum raised against CfaE as the primary antibody in immunoelectron microscopy (IEM), a pattern of decoration was found that definitively supports localization at the outermost tips of peritrichous CFA/I fimbriae.

EXAMPLE 3

Method for the Induction of Immunity to Conformationally Stable Class 5 Adhesin Construct

The adhesins, located on the distal tip of fimbriae of certain *E. coli* are the most important component for the induction of diarrheagenic *E. coli* bacterial immunity. However, fimbrial adhesins are inherently unstable and subject to degradation when devoid of their non-covalent linkage to major subunits fimbrial components. Therefore, improvements in conferring of protease resistance and conformational stability is important for production of maximally effective induction of B-cell activity capable of conferring anti-adhesive immunity against *E. coli*, including enterotoxigenic *E. coli*.

An aspect of this invention is the construction of stable polypeptide construct, as shown in Example 2. As taught in Example 1, protection against pathology caused by *E. coli* can be mediated by inhibition of colonization of bacteria by sterically hindering adhesion of fimbriae, and therefore bacteria, by induction of a specific B-cell response to adhesin polypeptide regions. Another aspect of this invention, therefore, is the induction of immunity by administration of a conformationally-stable polypeptide construct.

The construct comprises an antigenic fragment comprising an adhesin polypeptide sequence linked at the C-terminal regions to a linker that is itself operatively linked, at its C-terminal end, to a polypeptide of a major structural fimbrial subunit, such as CfaB. The antigenic fragment can be comprised of adhesin polypeptide sequences encoding any *E. coli* adhesin or adhesin fragment or alternatively polymers of adhesin polypeptides. Adhesins are selected from the group consisting of CfaE (SEQ ID NO 4), CsfD (SEQ ID NO 31), CsuD (SEQ ID NO 32), CooD (SEQ ID NO 33), CosD (SEQ ID NO 34), CsdD (SEQ ID NO 35), CsbD (SEQ ID NO 36 and SEQ ID NO 37) and CotD (SEQ ID NO 38).

The method for induction of anti-adhesin-mediated colonization of diarrheagenic bacteria contains the following steps:

- priming is by administration of immunogen containing said conformationally-stable adhesin polypeptide construct. Immunogen can be administered orally, nasally, subcutaneously, intradermally, transdermally, transcutaneously intramuscularly, or rectally. The range of a unit dose of immunogen is 50 μ g to 1 mg of immunogen. The immunogen is administered in any number of solutions with or without carrier protein or adjuvant or adsorbed into particles such as microspheres;
- Subsequent to a priming dose, 2 to 4 boosting doses are also administered with unit dose range of 50 μ g to 1 mg of immunogen in a buffered aqueous solution.

An alternative vaccine approach is the administration of the DNA construct described in Example 2 but inserted and expressed in host bacterial cells. The recombinant host cells can then be administered as a whole cell vaccine in order to confer immunity not only to the host cell but against the expressed ETEC recombinant adhesin polypeptides. Representative host cells include, but are not limited to *Escherichia coli*, members of the genus *Shigella*, members of the genus

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Campylobacter, members of the genus *Salmonella*, members of the genus *Vibrio* including *Vibrio cholerae*.

A method for the induction of whole cell immunity contains the following steps:

- a. administration of a priming dose comprising an adequate numbers of whole cell bacteria, selected from the group consisting of *Escherchia coli*, *Shigella* spp, *Campylobacter* spp, *Vibrio* spp and *Vibrio cholerae*, such that the expressed recombinant adhesin polypeptide is 50 µg to 1 mg per dose.
- b. Subsequent to priming dose, administration of 1 to 4 boosting doses of whole cell bacteria, selected from the group consisting of *Escherchia coli*, *Shigella* spp, *Campylobacter* spp, *Vibrio* spp and *Vibrio cholerae*, such that the expressed recombinant adhesin polypeptide is in the range of 50 µg to 1 mg per dose. Alternatively, the boosting doses can be immunogen containing said protease resistant adhesin peptide construct a unit dose range of 50 µg to 1 mg of immunogen in a buffered aqueous solution.

As a specific example in order to illustrate the method, the construct described in Example 2 was utilized to induce an immune response in mice. FIG. 9 shows IgG and IgA responses to homologous antigen in ELISA following either orogastric or intranasal administration of CfaE, CfaE plus mLT, CFA/I or CFA/I plus mLT. In FIG. 9, groups of mice (n=6) were administered three (3) doses at 2 week intervals of either fimbria (CFA/I) (250 µg), CFA/I (250 µg) plus mLT (mLT=*E. coli* heat labile toxin LTR192G) (10 µg), dscCfaE (250 µg) or dscCfaE (250 µg) plus mLT mLT=LTR 192G (10 µg). Serum was collected approximately 42 hours after the initial immunization. As illustrated in FIG. 9, CfaE or fimbria (CFA/I) induced a vigorous IgG and IgA response and significantly enhanced by the simultaneous administration of mLT. Interestingly, the simultaneous administration of mLT with CfaE or fimbria (CFA/I), intranasally or orogastrically, yielded a greater overall antibody response for CfaE than for CFA/I.

FIG. 10 illustrates antibody titers specific to either CfaE or CFA/I induced following administration of CfaE versus CFA/I, either with mLT. As in FIG. 9, groups of mice (n=6) were administered three (3) doses at 2 week intervals of either CFA/I (250 µg) plus mLT (mLT=*E. coli* heat labile toxin LTR192G) (10 µg) or dscCfaE (250 µg) plus mLT mLT=LTR 192G (10 µg). Following immunization, serum antibody titers were measured by ELISA using homologous antigen. FIGS. 10 (a) and (b) show antibody titers induced following orogastric administration of either CfaE plus mLT and FIGS. 10 (c) and (d) show antibody titers induced following intranasal administration. Following either orogastric or intranasal administration of CfaE and CFA/I plus mLT, immunization with dscCfaE resulted in a higher titer of specific IgG antibody response. These data indicate that dscCfaE is an effective, when administered at least via the intranasal and orogastric route, at inducing an immune response.

As illustrated in FIG. 10, dscCfaE can effectively induce a high titer of antibody. To ascertain if the antibody was functional, analysis of the serum antibody is illustrated in FIG. 11. FIG. 11 (a) shows the HAI titer of serum obtained following intranasal administration of either CFA/I or CfaE and FIG. 11 (b) shows the HAI response of serum obtained following orogastric administration. As illustrated by FIG. 11, immunization with CfaE induced much more robust inhibitory activity than CFA/I, regardless of the route of administration. The increased functional activity is correlated with the titer of anti-CfaE antibody represented in the serum. Collectively,

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these data illustrate that the dscCfaE construct is capable of inducing high titers of functional antibody.

EXAMPLE 4

Method for the Induction of Immunity to Class 5 Fimbriae Adhesin

An aspect of this invention is that the most important component of *E. coli* fimbriae for inducing an immune response against *E. coli* capable of effectively preventing bacterial pathology is adhesin (as taught in Example 1). These molecules are located on the distal tip of native fimbriae. It is important, therefore, to induce immunity, principally a B-cell response with concomitant production of immunoglobulin specific for adhesin molecule regions capable of inhibiting adhesin attachment to host cells (see inhibition of adhesin in example 1).

Immunoglobulin-mediated immunity can be effected by steric hindrance caused by binding at or near the active host-cell binding site or by binding to epitopes remote from adhesin host-cell binding site. A method for the induction of anti-adhesin mediated colonization of diarrheagenic bacteria contains the following steps:

- a. priming is by administration of immunogen comprising whole fimbriae containing adhesin. Alternatively, isolated fragments of fimbriae, containing adhesin or adhesin polypeptides alone, can be used rather than intact fimbriae. Immunogen can be administered orally, nasally, subcutaneously, intradermally, transdermally, transcutaneously intramuscularly, or rectally. The range of a unit dose of immunogen is 50 µg to 1 mg of immunogen. The immunogen is administered in any number of aqueous buffered solutions with or without carrier protein or adjuvant;
- b. Subsequent to a priming dose, 2 to 4 boosting doses are also administered with unit dose range of 50 µg to 1 mg of immunogen in a buffered aqueous solutions.

Referring to FIG. 9, either orogastric or intranasal administration of CFA/I, with or without the adjuvant mLT induced a significant serum IgG response following a three (3) dose regimen. As previously described, groups of mice (n=6) were administered three (3) doses at 2 week intervals of either CFA/I (250 µg), CFA/I (250 µg) plus mLT (mLT=LTR192G) (10 µg), dscCfaE (250 µg) or dscCfaE (250 µg) plus mLT mLT=LTR 192G (10 µg). Notwithstanding the robust antibody response following immunization with fimbria (i.e. CFA/I), as illustrated in FIG. 9 and FIG. 10, anti-CFA/I serum contained a modest anti-CfaE activity as illustrated in FIG. 10. Consistent with this observation, referring to FIG. 11, a significant HAI titer was also seen using the serum antibody obtain following CFA/I administration. Nevertheless, the antibody and HAI responses to CFA/I, which contains an adhesin tip, is much less than that obtained when stable CfaE (dscCfaE) is used as immunogen, as illustrated in FIG. 10 and FIG. 11.

EXAMPLE 5

Induction of Anti-ETEC Immunity Using an Anti-CS3 Construct

CS3 is composed of two distinct subunits, CstH and CstG (Savarino, unpublished). This conclusion is contrary to earlier published observations and conclusions (39, 40). Purified CS3 from wild type ETEC strain M424C1 (LTST-CS1+CS3-O6:H16) was resolved into two closely migrating protein

bands on SDS-PAGE, each with distinct N-terminal amino acid sequences. DNA sequence analysis of the M424C1 CS3 gene cluster revealed two contiguous open reading frames (ORFs) at the 3-prime end of the cluster that encode the proteins CstH and CstG whose N-terminal regions match exactly with the two experimentally derived N-terminal sequences of CS3 (Savarino S J, unpublished data). These two subunits share 46% similarity and appear to be present in purified fimbriae in a ratio of nearly 1:1.5, as compared to the estimated ratio of 1:1000 for the CfaE/CfaB minor and major subunits, respectively, of CFA/I (37).

By mutation and complementation experiments, we found that both CstH and CstG subunits are necessary for expression of CS3 fibrillae. Recombinant plasmids were engineered to express MBP fusions to the signal peptide-cleaved forms of CstH and CstG, and each was used to generate rabbit polyclonal antibodies. Preincubation of purified IgG and Fab fractions from the anti-MBPCstH but not anti-MBPCstG with wild type CS3-ETEC (strain WS2010A) inhibited bovine erythrocyte MRHA, the surrogate in vitro binding phenotype of CS3. We also engineered fusions of CstH and CstG to an intein carrier (41), and purified these passenger proteins by chitin affinity chromatography (New England Biolabs, Ipswich, Mass.) and in-column autocleavage at the intein-passenger protein junction. Rabbit polyclonal antisera generated against purified CstH but not CstG also exhibited hemagglutination inhibition (HAI) activity, corroborating the results observed with antibodies against the corresponding MBP fusions (see FIG. 12). In FIG. 12, reactivity to PCF039 fimbriae was included as a negative control. Our results support the contention that CstH is the actual binding subunit of CS3 and hence may serve as a precise vaccine target for generating anti-adhesive humoral immune responses.

Based on the available evidence indicating that CstH is the CS3 adhesin, we undertook efforts to engineer a stable CstH construct. As mentioned, we cloned CstH as a C-terminal fusion to intein (IMPACT-CN™ expression system, New England Biolabs™). This system offered reasonable yields and purity of CstH at the 1 L flask culture level. Scale-up to a 10 L fermentor resulted in high-level expression of the intein-CstH fusion product, however, was largely confined to the insoluble fraction after cell disruption, making this less suitable as a system for intermediate or large-scale production efforts. The untagged, mature form of CstH that we derived from use of this system did, however permitted protein characterization.

Native gel electrophoresis and size exclusion chromatography indicated that CstH self-assembles into oligomers by ordered, noncovalent interaction with a (with a mass range indicating formation of CstH 4-16mers). High resolution electron microscopy to demonstrated two distinct morphologic forms. CstH oligomers were observed as either globular or linear particles, and each type showed some variation in size and arrangement.

While CstH particle formation may confer some favorable immunologic properties, the apparent heterogeneity of such a preparation poses potential difficulties as it relates to developing a reproducible manufacturing process with defined end-product characteristics. Therefore, donor strand complementation was utilized in order to design stable CstH constructs.

The CS3 fibrillar assembly has been classified as a member of the classical chaperone-usher (CU) pathway based on the genetic relatedness of the CS3 periplasmic chaperone to the PapD superfamily (42). Interestingly, it falls into the FGL (F1-G1 long) subfamily, referring to a characteristic structural feature of the chaperone, which mediates assembly of

thin fibrillar or afimbrial adhesive organelles (43). Alignment of the N-terminal amino acid span of CstH with *Yersinia pestis* F1 capsule subunit reveals a common motif of alternating hydrophobic residues through amino acid 16 (with reference to the mature CstH polypeptide). This span of the F1 capsular subunit (CafI) functions as the donor strand, interacting with the CafIM chaperone and neighboring F1 protein subunits during capsular assembly and subunit articulation (44).

Reasoning that the corresponding CstH segment may function in a similar fashion, two in-cis donor strand complemented CstH constructs were engineered. The full-length CstH sequence (SEQ ID No. 19) contains a 22 amino acid signal peptide that is normally cleaved upon entry into the periplasm to give the mature CstH sequence (SEQ ID No. 23). The mature sequence also contains a 16 amino acid terminal β -strand disclosed in SEQ ID No. 20. FIG. 13 schematically illustrates the construct design. FIG. 13 (A) and FIG. 13 (C) illustrate the mature CstH amino acid sequence, but with the 22 amino acid leader sequence removed and a His-tag inserted. In FIG. 13 (A), a [His]₁₀ tag is inserted to the N-terminus of the mature CstH. In FIG. 13 (C), a [His]₆ tag is inserted to the C-terminal end of the mature CstH.

FIG. 13 (B) and (D) illustrate further modifications. FIG. 13 (B) illustrates the construct [His]₁₀dsc₁₆CstH, disclosed in SEQ ID No. 21. [His]₁₀dsc₁₆CstH contains an N-terminal His₁₀, as in FIG. 13 (A) but with a short hairpin linker (SEQ ID No 1, 2 or 3) fused to the C-terminal end of the mature CstH which is in-turn fused at its C-terminal end to a duplicated donor strand derived from the first 16 amino acids from the CstH terminus disclosed in SEQ ID No. 20. FIG. 13 (D) schematically illustrates dsc₁₆CstH[His]₆, which is disclosed as SEQ ID No. 22. This construct contains a His-tag at the C-terminus, verses at the N-terminal end, as in [His]₁₀dsc₁₆CstH. The two amino acids between the C-terminal end of the in cis donor strand and the His-tag are derived from the expression vector multicloning side coding sequence. The [His]₁₀dsc₁₆CstH construct was inserted into the T7 expression plasmid pET 19 and is referred to pET19/[His]₁₀dsc₁₆CstH. Similarly, the dsc₁₆CstH[His]₆ construct was inserted into pET24 and is referred to as pET24/dsc₁₆CstH [His]₆. The dsc₁₆CstH[His]₆ construct exhibited high solubility.

Electrophoretic analysis demonstrated that the expressed construct exhibited monomeric characteristics as illustrated in FIG. 14. In FIG. 14 (A), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) shows a clear prominent band. Western blot analysis using anti-CstH and anti-CS3 (FIGS. 14 (B) and (C)), respectively, also show a clearly prominent monomeric band.

The CS3 construct is contemplated to be utilized by a method similar to that described in Example 3. Therefore, induction of immunity using dsc₁₆CstH-[His]₆, or other variants, is by the method comprising the steps:

- a. priming is by administration of the [His]₁₀dsc₁₆CstH or dsc₁₆CstH-[His]₆ (i.e. SEQ ID No. 21 or SEQ ID No. 22) immunogen or variants (as illustrated in FIG. 13) containing said conformationally-stable adhesin polypeptide construct. Immunogen can be administered orally, nasally, subcutaneously, intradermally, transdermally, transcutaneously intramuscularly, or rectally. The range of a unit dose of immunogen is 50 μ g to 1 mg of immunogen. The immunogen is administered in any number of solutions with or without carrier protein or adjuvant or adsorbed into particles such as microspheres;

b. Subsequent to a priming dose, 2 to 4 boosting doses are also administered with unit dose range of 50 µg to 1 mg of immunogen in a buffered aqueous solution.

The CstH construct can also be used expressed in host bacterial cells including *Escherichia coli*, members of the genus *Shigella*, members of the genus *Campylobacter*, members of the genus *Salmonella*, members of the genus *Vibrio* including *Vibrio cholerae* as described for the class 5 adhesin construct in Example 3.

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- Having described the invention, one of skill in the art will appreciate in the appended claims that many modifications and variations of the present invention are possible in light of the above teachings. It is therefore, to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

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      20      25      30
Thr Asn Thr Ile Gly Pro His Asp Arg Gly Gly Ser Ser Pro Ile Tyr
      35      40      45
Asn Ile Leu Asn Ser Tyr Leu Thr Ala Tyr Asn Gly Ser His His Leu
      50      55      60
Tyr Asp Arg Met Ser Phe Leu Cys Leu Ser Ser Gln Asn Thr Leu Asn
      65      70      75      80
Gly Ala Cys Pro Ser Ser Asp Ala Pro Gly Thr Ala Thr Ile Asp Gly
      85      90      95
Glu Thr Asn Ile Thr Leu Gln Phe Thr Glu Lys Arg Ser Leu Ile Lys
      100     105     110
Arg Glu Leu Gln Ile Lys Gly Tyr Lys Gln Phe Leu Phe Lys Asn Ala
      115     120     125
Asn Cys Pro Ser Lys Leu Ala Leu Asn Ser Ser His Phe Gln Cys Asn
      130     135     140
Arg Glu Gln Ala Ser Gly Ala Thr Leu Ser Leu Tyr Ile Pro Ala Gly
      145     150     155     160
Glu Leu Asn Lys Leu Pro Phe Gly Gly Val Trp Asn Ala Val Leu Lys
      165     170     175
Leu Asn Val Lys Arg Arg Tyr Asp Thr Thr Tyr Gly Thr Tyr Thr Ile
      180     185     190
Asn Ile Thr Val Asn Leu Thr Asp Lys Gly Asn Ile Gln Ile Trp Leu
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Pro Gln Phe Lys Ser Asn Ala Arg Val Asp Leu Asn Leu Arg Pro Thr
      210     215     220
Gly Gly Gly Thr Tyr Ile Gly Arg Asn Ser Val Asp Met Cys Phe Tyr
      225     230     235     240
Asp Gly Tyr Ser Thr Asn Ser Ser Ser Leu Glu Ile Arg Phe Gln Asp
      245     250     255
Asp Asn Ser Lys Ser Asp Gly Lys Phe Tyr Leu Lys Lys Ile Asn Asp
      260     265     270
Asp Ser Lys Glu Leu Val Tyr Thr Leu Ser Leu Leu Ala Gly Lys
      275     280     285
Asn Leu Thr Pro Thr Asn Gly Gln Ala Leu Asn Ile Asn Thr Ala Ser
      290     295     300
Leu Glu Thr Asn Trp Asn Arg Ile Thr Ala Val Thr Met Pro Glu Ile
      305     310     315     320
Ser Val Pro Val Leu Cys Trp Pro Gly Arg Leu Gln Leu Asp Ala Lys
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Ser Val Asp Pro Ala Ile Asp Leu Leu Gln Ala Asp Gly Asn Ala Leu
      35           40           45

Pro Ser Ala Val Lys Leu Ala Tyr Ser Pro Ala Ser Lys Thr Phe Glu
      50           55           60

Ser Tyr Arg Val Met Thr Gln Val His Thr Asn Asp Ala Thr Lys Lys
      65           70           75           80

Val Ile Val Lys Leu Ala Asp Thr Pro Gln Leu Thr Asp Val Leu Asn
      85           90           95

Ser Thr Val Gln Met Pro Ile Ser Val Ser Trp Gly Gly Gln Val Leu
      100          105          110

Ser Thr Thr Ala Lys Glu Phe Glu Ala Ala Ala Leu Gly Tyr Ser Ala
      115          120          125

Ser Gly Val Asn Gly Val Ser Ser Ser Gln Glu Leu Val Ile Ser Ala
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Ser Val Asp Pro Val Ile Asp Leu Leu Gln Ala Asp Gly Asn Ala Leu
      35           40           45

Pro Ser Ala Val Lys Leu Ala Tyr Ser Pro Ala Ser Lys Thr Phe Glu
      50           55           60

Ser Tyr Arg Val Met Thr Gln Val His Thr Asn Asp Ala Thr Lys Lys
      65           70           75           80

Val Ile Val Lys Leu Ala Asp Thr Pro Gln Leu Thr Asp Val Leu Asn
      85           90           95

Ser Thr Val Gln Met Pro Ile Ser Val Ser Trp Gly Gly Gln Val Leu
      100          105          110

Ser Thr Thr Ala Lys Glu Phe Glu Ala Ala Ala Leu Gly Tyr Ser Ala
      115          120          125

Ser Gly Val Asn Gly Val Ser Ser Ser Gln Glu Leu Val Ile Ser Ala
      130          135          140

Ala Pro Lys Thr Ala Gly Thr Ala Pro Thr Ala Gly Asn Tyr Ser Gly
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 Leu Leu Gln Ser
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Leu Leu Gln Ala
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Leu Leu Gln Ala
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Leu Met Gln Ser
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Leu Leu Gln Ser
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20           25           30
Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp Ala Thr Trp Ala Pro
35           40           45
Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val Ser Asn Thr Leu Val
50           55           60
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65           70           75           80
Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly Thr Val Thr Phe Ala
85           90           95
His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr Thr Ile Ser Thr Asp
100          105          110
Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly Asn Thr Ile Val Lys
115          120          125
Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu Pro Leu Lys Phe Ile
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Ile Thr Ile Thr Ser Thr Ile Lys
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20           25           30
Glu Leu Ala Leu Asn Val Leu Ser Pro Ala Ala Leu Asp Ala Thr Trp
35           40           45
Ala Pro Gln Asp Asn Leu Thr Leu Ser Asn Thr Gly Val Ser Asn Thr
50           55           60
Leu Val Gly Val Leu Thr Leu Ser Asn Thr Ser Ile Asp Thr Val Ser
65           70           75           80
Ile Ala Ser Thr Asn Val Ser Asp Thr Ser Lys Asn Gly Thr Val Thr
85           90           95
Phe Ala His Glu Thr Asn Asn Ser Ala Ser Phe Ala Thr Thr Ile Ser

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100	105	110
Thr Asp Asn Ala Asn Ile Thr Leu Asp Lys Asn Ala Gly Asn Thr Ile		
115	120	125
Val Lys Thr Thr Asn Gly Ser Gln Leu Pro Thr Asn Leu Pro Leu Lys		
130	135	140
Phe Ile Thr Thr Glu Gly Asn Glu His Leu Val Ser Gly Asn Tyr Arg		
145	150	155
Ala Asn Ile Thr Ile Thr Ser Thr Ile Lys Asp Asn Lys Gln Ala Ala		
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Leu Ser Asn Thr Gly Val Ser Asn Thr Leu Val Gly Val Leu Thr Leu		
35	40	45
Ser Asn Thr Ser Ile Asp Thr Val Ser Ile Ala Ser Thr Asn Val Ser		
50	55	60
Asp Thr Ser Lys Asn Gly Thr Val Thr Phe Ala His Glu Thr Asn Asn		
65	70	80
Ser Ala Ser Phe Ala Thr Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr		
85	90	95
Leu Asp Lys Asn Ala Gly Asn Thr Ile Val Lys Thr Thr Asn Gly Ser		
100	105	110
Gln Leu Pro Thr Asn Leu Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn		
115	120	125
Glu His Leu Val Ser Gly Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser		
130	135	140
Thr Ile Lys Asp Asn Lys Gln Ala Ala Gly Pro Thr Leu Thr Lys Glu		
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20	25	30
Ser Asn Thr Gly Val Ser Asn Thr Leu Val Gly Val Leu Thr Leu Ser		
35	40	45
Asn Thr Ser Ile Asp Thr Val Ser Ile Ala Ser Thr Asn Val Ser Asp		
50	55	60
Thr Ser Lys Asn Gly Thr Val Thr Phe Ala His Glu Thr Asn Asn Ser		

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65	70	75	80
Ala Ser Phe Ala Thr Thr Ile Ser Thr Asp Asn Ala Asn Ile Thr Leu			
	85	90	95
Asp Lys Asn Ala Gly Asn Thr Ile Val Lys Thr Thr Asn Gly Ser Gln			
	100	105	110
Leu Pro Thr Asn Leu Pro Leu Lys Phe Ile Thr Thr Glu Gly Asn Glu			
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His Leu Val Ser Gly Asn Tyr Arg Ala Asn Ile Thr Ile Thr Ser Thr			
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Ile Lys			
145			

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Leu Leu Gln Ala
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<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 31

Met Asn Lys Ile Leu Phe Ile Phe Thr Leu Phe Phe Ser Ser Val Leu
 1 5 10 15
 Phe Thr Phe Ala Val Ser Ala Asp Lys Ile Pro Gly Asp Glu Ser Ile
 20 25 30
 Thr Asn Ile Phe Gly Pro Arg Asp Arg Asn Glu Ser Ser Pro Lys His
 35 40 45
 Asn Ile Leu Asn Asn His Ile Thr Ala Tyr Ser Glu Ser His Thr Leu
 50 55 60
 Tyr Asp Arg Met Thr Phe Leu Cys Leu Ser Ser His Asn Thr Leu Asn
 65 70 75 80
 Gly Ala Cys Pro Thr Ser Glu Asn Pro Ser Ser Ser Val Ser Gly
 85 90 95
 Glu Thr Asn Ile Thr Leu Gln Phe Thr Glu Lys Arg Ser Leu Ile Lys
 100 105 110
 Arg Glu Leu Gln Ile Lys Gly Tyr Lys Gln Leu Leu Phe Lys Ser Val
 115 120 125
 Asn Cys Pro Ser Gly Leu Thr Leu Asn Ser Ala His Phe Asn Cys Asn
 130 135 140
 Lys Asn Ala Ala Ser Gly Ala Ser Leu Tyr Leu Tyr Ile Pro Ala Gly
 145 150 155 160
 Glu Leu Lys Asn Leu Pro Phe Gly Gly Ile Trp Asp Ala Thr Leu Lys
 165 170 175
 Leu Arg Val Lys Arg Arg Tyr Ser Glu Thr Tyr Gly Thr Tyr Thr Ile
 180 185 190

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Asn Ile Thr Ile Lys Leu Thr Asp Lys Gly Asn Ile Gln Ile Trp Leu
  195                200                205

Pro Gln Phe Lys Ser Asp Ala Arg Val Asp Leu Asn Leu Arg Pro Thr
  210                215                220

Gly Gly Gly Thr Tyr Ile Gly Arg Asn Ser Val Asp Met Cys Phe Tyr
  225                230                235                240

Asp Gly Tyr Ser Thr Asn Ser Ser Ser Leu Glu Ile Arg Phe Gln Asp
  245                250                255

Asn Asn Pro Lys Ser Asp Gly Lys Phe Tyr Leu Arg Lys Ile Asn Asp
  260                265                270

Asp Thr Lys Glu Ile Ala Tyr Thr Leu Ser Leu Leu Leu Ala Gly Lys
  275                280                285

Ser Leu Thr Pro Thr Asn Gly Thr Ser Leu Asn Ile Ala Asp Ala Ala
  290                295                300

Ser Leu Glu Thr Asn Trp Asn Arg Ile Thr Ala Val Thr Met Pro Glu
  305                310                315                320

Ile Ser Val Pro Val Leu Cys Trp Pro Gly Arg Leu Gln Leu Asp Ala
  325                330                335

Lys Val Glu Asn Pro Glu Ala Gly Gln Tyr Met Gly Asn Ile Asn Val
  340                345                350

Thr Phe Thr Pro Ser Ser Gln Thr Leu
  355                360

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<210> SEQ ID NO 32

<211> LENGTH: 361

<212> TYPE: PRT

<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 32

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Met Asn Lys Ile Leu Phe Ile Phe Thr Leu Phe Phe Ser Ser Val Leu
  1          5          10          15

Phe Thr Phe Ala Val Ser Ala Asp Lys Ile Pro Gly Asp Glu Asn Ile
  20          25          30

Thr Asn Ile Phe Gly Pro Arg Asp Arg Asn Glu Ser Ser Pro Lys His
  35          40          45

Asn Ile Leu Asn Asp Tyr Ile Thr Ala Tyr Ser Glu Ser His Thr Leu
  50          55          60

Tyr Asp Arg Met Ile Phe Leu Cys Leu Ser Ser Gln Asn Thr Leu Asn
  65          70          75          80

Gly Ala Cys Pro Thr Ser Glu Asn Pro Ser Ser Ser Ser Val Ser Gly
  85          90          95

Glu Thr Asn Ile Thr Leu Gln Phe Thr Glu Lys Arg Ser Leu Ile Lys
  100         105         110

Arg Glu Leu Gln Ile Lys Gly Tyr Lys Arg Leu Leu Phe Lys Gly Ala
  115         120         125

Asn Cys Pro Ser Tyr Leu Thr Leu Asn Ser Ala His Tyr Thr Cys Asn
  130         135         140

Arg Asn Ser Ala Ser Gly Ala Ser Leu Tyr Leu Tyr Ile Pro Ala Gly
  145         150         155         160

Glu Leu Lys Asn Leu Pro Phe Gly Gly Ile Trp Asp Ala Thr Leu Lys
  165         170         175

Leu Arg Val Lys Arg Arg Tyr Asp Gln Thr Tyr Gly Thr Tyr Thr Ile
  180         185         190

Asn Ile Thr Val Lys Leu Thr Asp Lys Gly Asn Ile Gln Ile Trp Leu

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195	200	205
Pro Gln Phe Lys Ser Asp	Ala Arg Val Asp Leu	Asn Leu Arg Pro Thr
210	215	220
Gly Gly Gly Thr Tyr Ile	Gly Arg Asn Ser Val	Asp Met Cys Phe Tyr
225	230	235
Asp Gly Tyr Ser Thr Asn	Ser Ser Leu Glu Leu	Arg Phe Gln Asp
245	250	255
Asn Asn Pro Lys Ser Asp	Gly Lys Phe Tyr Leu	Arg Lys Ile Asn Asp
260	265	270
Asp Thr Lys Glu Ile Ala	Tyr Thr Leu Ser Leu	Leu Leu Ala Gly Lys
275	280	285
Ser Leu Thr Pro Thr Asn	Gly Thr Ser Leu Asn	Ile Ala Asp Ala Ala
290	295	300
Ser Leu Glu Ile Asn Trp	Asn Arg Ile Thr Ala	Val Thr Met Pro Glu
305	310	315
Ile Ser Val Pro Val Leu	Cys Trp Pro Gly Arg	Leu Gln Leu Asp Ala
325	330	335
Lys Val Glu Asn Pro Glu	Ala Gly Gln Tyr Met	Gly Asn Ile Asn Ile
340	345	350
Thr Phe Thr Pro Ser Ser	Gln Thr Leu	
355	360	
<210> SEQ ID NO 33		
<211> LENGTH: 363		
<212> TYPE: PRT		
<213> ORGANISM: Escherichia coli		
<400> SEQUENCE: 33		
Met Lys Lys Ile Phe Ile	Phe Leu Ser Ile Ile	Phe Ser Ala Val Val
1	5	10
Ser Ala Gly Arg Tyr Pro	Glu Thr Thr Val Gly	Asn Leu Thr Lys Ser
20	25	30
Phe Gln Ala Pro Arg Leu	Asp Arg Ser Val Gln	Ser Pro Ile Tyr Asn
35	40	45
Ile Phe Thr Asn His Val	Ala Gly Tyr Ser Leu	Ser His Ser Leu Tyr
50	55	60
Asp Arg Ile Val Phe Leu	Cys Thr Ser Ser Ser	Asn Pro Val Asn Gly
65	70	75
Ala Cys Pro Thr Ile Gly	Thr Ser Gly Val Gln	Tyr Gly Thr Thr Thr
85	90	95
Ile Thr Leu Gln Phe Thr	Glu Lys Arg Ser Leu	Ile Lys Arg Asn Ile
100	105	110
Asn Leu Ala Gly Asn Lys	Lys Pro Ile Trp Glu	Asn Gln Ser Cys Asp
115	120	125
Phe Ser Asn Leu Met Val	Leu Asn Ser Lys Ser	Trp Ser Cys Gly Ala
130	135	140
His Gly Asn Ala Asn Gly	Thr Leu Leu Asn Leu	Tyr Ile Pro Ala Gly
145	150	155
Glu Ile Asn Lys Leu Pro	Phe Gly Gly Ile Trp	Glu Ala Thr Leu Ile
165	170	175
Leu Arg Leu Ser Arg Tyr	Gly Glu Val Ser Ser	Thr His Tyr Gly Asn
180	185	190
Tyr Thr Val Asn Ile Thr	Val Asp Leu Thr Asp	Lys Gly Asn Ile Gln
195	200	205

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Val	Trp	Leu	Pro	Gly	Phe	His	Ser	Asn	Pro	Arg	Val	Asp	Leu	Asn	Leu
210						215					220				
Arg	Pro	Ile	Gly	Asn	Tyr	Lys	Tyr	Ser	Gly	Ser	Asn	Ser	Leu	Asp	Met
225				230					235						240
Cys	Phe	Tyr	Asp	Gly	Tyr	Ser	Thr	Asn	Ser	Asp	Ser	Met	Val	Ile	Lys
			245					250						255	
Phe	Gln	Asp	Asp	Asn	Pro	Thr	Asn	Ser	Ser	Glu	Tyr	Asn	Leu	Tyr	Lys
		260					265						270		
Ile	Gly	Gly	Thr	Glu	Lys	Leu	Pro	Tyr	Ala	Val	Ser	Leu	Leu	Met	Gly
	275					280						285			
Glu	Lys	Ile	Phe	Tyr	Pro	Val	Asn	Gly	Gln	Ser	Phe	Thr	Ile	Asn	Asp
290					295						300				
Ser	Ser	Val	Leu	Glu	Thr	Asn	Trp	Asn	Arg	Val	Thr	Ala	Val	Ala	Met
305				310					315						320
Pro	Glu	Val	Asn	Val	Pro	Val	Leu	Cys	Trp	Pro	Ala	Arg	Leu	Leu	Leu
			325					330						335	
Asn	Ala	Asp	Val	Asn	Ala	Pro	Asp	Ala	Gly	Gln	Tyr	Ser	Gly	Gln	Ile
		340					345						350		
Tyr	Ile	Thr	Phe	Thr	Pro	Ser	Val	Glu	Asn	Leu					
	355					360									

<210> SEQ ID NO 34
 <211> LENGTH: 363
 <212> TYPE: PRT
 <213> ORGANISM: Escherichia coli

<400> SEQUENCE: 34

Met	Lys	Lys	Ile	Phe	Ile	Phe	Leu	Ser	Ile	Ile	Phe	Ser	Ala	Val	Val
1			5					10					15		
Ser	Ala	Gly	Arg	Tyr	Pro	Glu	Thr	Thr	Val	Gly	Asn	Leu	Thr	Lys	Ser
	20						25					30			
Phe	Gln	Ala	Pro	Arg	Leu	Asp	Arg	Ser	Val	Gln	Ser	Pro	Ile	Tyr	Asn
	35				40						45				
Ile	Phe	Thr	Asn	His	Val	Ala	Gly	Tyr	Ser	Leu	Ser	His	Arg	Leu	Tyr
	50			55						60					
Asp	Arg	Ile	Val	Phe	Val	Cys	Thr	Ser	Ser	Ser	Asn	Pro	Val	Asn	Gly
65			70						75					80	
Ala	Cys	Pro	Thr	Ile	Gly	Thr	Ser	Gly	Val	Glu	Tyr	Gly	Thr	Thr	Thr
		85						90					95		
Ile	Thr	Leu	Gln	Phe	Thr	Glu	Lys	Arg	Ser	Leu	Ile	Lys	Arg	Asn	Ile
		100					105						110		
Asn	Leu	Ala	Gly	Asn	Lys	Lys	Pro	Ile	Trp	Glu	Asn	Gln	Ser	Cys	Asp
	115					120						125			
Phe	Ser	Asn	Leu	Met	Val	Leu	Asn	Ser	Lys	Ser	Trp	Ser	Cys	Gly	Ala
	130				135						140				
Gln	Gly	Asn	Ala	Asn	Gly	Thr	Leu	Leu	Asn	Leu	Tyr	Ile	Pro	Ala	Gly
145				150					155					160	
Glu	Ile	Asn	Lys	Leu	Pro	Phe	Gly	Gly	Ile	Trp	Glu	Ala	Thr	Leu	Ile
		165						170						175	
Leu	Arg	Leu	Ser	Arg	Tyr	Gly	Glu	Val	Ser	Ser	Thr	His	Tyr	Gly	Asn
		180					185						190		
Tyr	Thr	Val	Asn	Ile	Thr	Val	Asp	Leu	Thr	Asp	Lys	Gly	Asn	Ile	Gln
	195					200					205				
Val	Trp	Leu	Pro	Gly	Phe	His	Ser	Asn	Pro	Arg	Val	Asp	Leu	Asn	Leu
210					215						220				

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His Pro Ile Gly Asn Tyr Lys Tyr Ser Gly Ser Asn Ser Leu Asp Met
 225 230 235 240
 Cys Phe Tyr Asp Gly Tyr Ser Thr Asn Ser Asp Ser Met Val Ile Lys
 245 250 255
 Phe Gln Asp Asp Asn Pro Thr Asn Ser Ser Glu Tyr Asn Leu Tyr Lys
 260 265 270
 Arg Gly Gly Thr Glu Lys Leu Pro Tyr Ala Val Ser Leu Leu Met Gly
 275 280 285
 Gly Lys Ile Phe Tyr Pro Val Asn Gly Gln Ser Phe Thr Ile Asn Asp
 290 295 300
 Ser Ser Val Leu Glu Thr Asn Trp Asn Arg Val Thr Ala Val Ala Met
 305 310 315 320
 Pro Glu Val Asn Val Pro Val Leu Cys Trp Pro Ala Arg Leu Leu Leu
 325 330 335
 Asn Ala Asp Val Asn Ala Pro Asp Ala Gly Gln Tyr Ser Gly Gln Ile
 340 345 350
 Tyr Ile Thr Phe Thr Pro Ser Val Glu Asn Leu
 355 360

<210> SEQ ID NO 35
 <211> LENGTH: 363
 <212> TYPE: PRT
 <213> ORGANISM: Escherichia coli

<400> SEQUENCE: 35

Met Lys Lys Ile Phe Ile Phe Leu Ser Ile Ile Phe Ser Ala Val Val
 1 5 10 15
 Ser Ala Gly Arg Tyr Pro Glu Thr Thr Val Gly Asn Leu Thr Lys Ser
 20 25 30
 Phe Gln Ala Pro Arg Leu Asp Arg Ser Val Gln Ser Pro Ile Tyr Asn
 35 40 45
 Ile Phe Thr Asn His Val Ala Gly Tyr Ser Leu Ser His Arg Leu Tyr
 50 55 60
 Asp Arg Ile Val Phe Val Cys Thr Ser Ser Ser Asn Pro Val Asn Gly
 65 70 75 80
 Ala Cys Pro Thr Ile Gly Thr Ser Gly Val Glu Tyr Gly Thr Thr Thr
 85 90 95
 Ile Thr Leu Gln Phe Thr Glu Lys Arg Ser Leu Ile Lys Arg Asn Ile
 100 105 110
 Asn Leu Ala Gly Asn Lys Lys Pro Ile Trp Glu Asn Gln Ser Cys Asp
 115 120 125
 Phe Ser Asn Leu Met Val Leu Asn Ser Lys Ser Trp Ser Cys Gly Ala
 130 135 140
 Gln Gly Asn Ala Asn Gly Thr Leu Leu Asn Leu Tyr Ile Pro Ala Gly
 145 150 155 160
 Glu Ile Asn Lys Leu Pro Phe Gly Gly Ile Trp Glu Ala Thr Leu Ile
 165 170 175
 Leu Arg Leu Ser Arg Tyr Gly Glu Val Ser Ser Thr His Tyr Gly Asn
 180 185 190
 Tyr Thr Val Asn Ile Thr Val Asp Leu Thr Asp Lys Gly Asn Ile Gln
 195 200 205
 Val Trp Leu Pro Gly Phe His Ser Asn Pro Arg Val Asp Leu Asn Leu
 210 215 220
 His Pro Ile Gly Asn Tyr Lys Tyr Ser Gly Ser Asn Ser Leu Asp Met

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225	230	235	240
Cys Phe Tyr Asp Gly Tyr Ser Thr Asn Ser Asp Ser Met Val Ile Lys	245	250	255
Phe Gln Asp Asp Asn Pro Thr Asn Ser Ser Glu Tyr Asn Leu Tyr Lys	260	265	270
Arg Gly Gly Thr Glu Lys Leu Pro Tyr Ala Val Ser Leu Leu Met Gly	275	280	285
Gly Lys Ile Phe Tyr Pro Val Asn Gly Gln Ser Phe Thr Ile Asn Asp	290	295	300
Ser Ser Val Leu Glu Thr Asn Trp Asn Arg Val Thr Ala Val Ala Met	305	310	315
Pro Glu Val Asn Val Pro Val Leu Cys Trp Pro Ala Arg Leu Leu Leu	325	330	335
Asn Ala Asp Val Asn Ala Pro Asp Ala Gly Gln Tyr Ser Gly Gln Ile	340	345	350
Tyr Ile Thr Phe Thr Pro Ser Val Glu Asn Leu	355	360	

<210> SEQ ID NO 36
 <211> LENGTH: 363
 <212> TYPE: PRT
 <213> ORGANISM: Escherichia coli

<400> SEQUENCE: 36

Met Lys Lys Ile Phe Ile Phe Leu Ser Ile Ile Phe Ser Ala Val Val	1	5	10	15
Ser Ala Gly Arg Tyr Pro Glu Thr Thr Val Gly Asn Leu Thr Lys Ser	20	25	30	
Phe Gln Ala Pro Arg Gln Asp Arg Ser Val Gln Ser Pro Ile Tyr Asn	35	40	45	
Ile Phe Thr Asn His Val Ala Gly Tyr Ser Leu Ser His Asn Leu Tyr	50	55	60	
Asp Arg Ile Val Phe Leu Cys Thr Ser Ser Ser Asn Pro Val Asn Gly	65	70	75	80
Ala Cys Pro Thr Leu Gly Thr Ser Gly Val Gln Tyr Gly Thr Thr Thr	85	90	95	
Ile Thr Leu Gln Phe Thr Glu Lys Arg Ser Leu Ile Lys Arg Asn Ile	100	105	110	
Asn Leu Ala Gly Asn Lys Lys Pro Ile Trp Glu Asn Gln Ser Cys Asp	115	120	125	
Thr Ser Asn Leu Met Val Leu Asn Ser Lys Ser Trp Ser Cys Gly His	130	135	140	
Tyr Gly Asn Ala Asn Gly Thr Leu Leu Asn Leu Tyr Ile Pro Ala Gly	145	150	155	160
Glu Ile Asn Lys Leu Pro Phe Gly Gly Ile Trp Glu Ala Thr Leu Ile	165	170	175	
Leu Arg Leu Ser Arg Tyr Gly Glu Val Ser Ser Thr His Tyr Gly Asn	180	185	190	
Tyr Thr Val Asn Ile Thr Val Asp Leu Thr Asp Lys Gly Asn Ile Gln	195	200	205	
Val Trp Leu Pro Gly Phe His Ser Asn Pro Arg Val Asp Leu Asn Leu	210	215	220	
His Pro Ile Gly Asn Tyr Lys Tyr Ser Gly Ser Asn Ser Leu Asp Met	225	230	235	240

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Phe Gln Asp Asp Asn Pro Thr Asn Ser Ser Glu Tyr Asn Leu Tyr Lys
 260 265 270
 Arg Gly Gly Thr Glu Lys Leu Pro Tyr Ala Val Ser Leu Leu Met Gly
 275 280 285
 Gly Lys Ile Phe Tyr Pro Val Asn Gly Gln Ser Phe Thr Ile Asn Asp
 290 295 300
 Ser Ser Val Leu Glu Thr Asn Trp Asn Arg Val Thr Ala Val Ala Met
 305 310 315 320
 Pro Glu Val Asn Val Pro Val Leu Cys Trp Pro Ala Arg Leu Leu Leu
 325 330 335
 Asn Ala Asp Val Asn Ala Pro Asp Ala Gly Gln Tyr Ser Gly Gln Ile
 340 345 350
 Tyr Ile Thr Phe Thr Pro Ser Val Glu Asn Leu
 355 360

<210> SEQ ID NO 38

<211> LENGTH: 355

<212> TYPE: PRT

<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 38

Met Phe Leu Cys Ser Gln Val Tyr Gly Gln Ser Trp His Thr Asn Val
 1 5 10 15
 Glu Ala Gly Ser Ile Asn Lys Thr Glu Ser Ile Gly Pro Ile Asp Arg
 20 25 30
 Ser Ala Ala Ala Ser Tyr Pro Ala His Tyr Ile Phe His Glu His Val
 35 40 45
 Ala Gly Tyr Asn Lys Asp His Ser Leu Phe Asp Arg Met Thr Phe Leu
 50 55 60
 Cys Met Ser Ser Thr Asp Ala Ser Lys Gly Ala Cys Pro Thr Gly Glu
 65 70 75 80
 Asn Ser Lys Ser Ser Gln Gly Glu Thr Asn Ile Lys Leu Ile Phe Thr
 85 90 95
 Glu Lys Lys Ser Leu Ala Arg Lys Thr Leu Asn Leu Lys Gly Tyr Lys
 100 105 110
 Arg Phe Leu Tyr Glu Ser Asp Arg Cys Ile His Tyr Val Asp Lys Met
 115 120 125
 Asn Leu Asn Ser His Thr Val Lys Cys Val Gly Ser Phe Thr Arg Gly
 130 135 140
 Val Asp Phe Thr Leu Tyr Ile Pro Gln Gly Glu Ile Asp Gly Leu Leu
 145 150 155 160
 Thr Gly Gly Ile Trp Glu Ala Thr Leu Glu Leu Arg Val Lys Arg His
 165 170 175
 Tyr Asp Tyr Asn His Gly Thr Tyr Lys Val Asn Ile Thr Val Asp Leu
 180 185 190
 Thr Asp Lys Gly Asn Ile Gln Val Trp Thr Pro Lys Phe His Ser Asp
 195 200 205
 Pro Arg Ile Asp Leu Asn Leu Arg Pro Glu Gly Asn Gly Lys Tyr Ser
 210 215 220
 Gly Ser Asn Val Leu Glu Met Cys Leu Tyr Asp Gly Tyr Ser Thr His
 225 230 235 240
 Ser Gln Ser Ile Glu Met Arg Phe Gln Asp Asp Ser Gln Thr Gly Asn
 245 250 255
 Asn Glu Tyr Asn Leu Ile Lys Thr Gly Glu Pro Leu Lys Lys Leu Pro

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260							265					270				
Tyr	Lys	Leu	Ser	Leu	Leu	Leu	Gly	Gly	Arg	Glu	Phe	Tyr	Pro	Asn	Asn	
275							280					285				
Gly	Glu	Ala	Phe	Thr	Ile	Asn	Asp	Thr	Ser	Ser	Leu	Phe	Ile	Asn	Trp	
290							295					300				
Asn	Arg	Ile	Lys	Ser	Val	Ser	Leu	Pro	Gln	Ile	Ser	Ile	Pro	Val	Leu	
305							310					315				
Cys	Trp	Pro	Ala	Asn	Leu	Thr	Phe	Met	Ser	Glu	Leu	Asn	Asn	Pro	Glu	
325							330					335				
Ala	Gly	Glu	Tyr	Ser	Gly	Ile	Leu	Asn	Val	Thr	Phe	Thr	Pro	Ser	Ser	
340							345					350				
Ser	Ser	Leu														
355																

- What is claimed is:
1. An immunogenic composition comprising CfaE or a fragment thereof linked at its C-terminal end to a polypeptide linker which is linked at the C-terminal end of said linker to CfaB or a fragment thereof.
 2. The immunogenic composition of claim 1, wherein said linker comprises the amino acid sequence of SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3 or fragments thereof.
 3. The immunogenic composition of claim 1, wherein said CfaE comprises the amino acid sequence of SEQ ID No. 4 or a fragment thereof.
 4. The immunogenic composition of claim 1, wherein said CfaB or a fragment thereof comprises a donor β -strand, ²⁰ wherein said donor β -strand comprises at least the first 12 amino acids of the major CfaB.
 5. The immunogenic composition of claim 1, wherein said CfaB comprises the amino acid sequence of SEQ ID No. 5 or SEQ ID No. 6 or an antigenic fragment thereof.
 6. The immunogenic composition of claim 1, wherein the ²⁵ fragment of CfaB has the amino acid sequence of SEQ ID No. 7.
 7. The immunogenic composition of claim 6, wherein the ³⁰ fragment of CfaB has an amino acid sequence comprising between the first 12 and the first 19 amino acids of SEQ ID No. 7.
- * * * * *